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LECITHIN AND STREPTOCOCCAL HÆMOLYSIN.

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IN a previous paper (Gordon, 1927) it was shown that the removal of the ether-soluble fraction of bouillon did not reduce the value of the medium for streptolysin formation. An extension of this work to the effect of adding lipoid substances, in particular lecithin, is here reported.

A streptococcus with marked hæmolytic properties was inoculated to 1% peptone bouillon and the culture was incubated for 18 hours. A good growth was obtained. As a test for hæmolysin production 0·1 c.c. of this culture added to 1 c.c. of 3% ox red blood-corpuscles gave complete hæmolysis in 2 hours at 37°C. If lecithin (ovolecithin, British Drug Houses) in various concentrations, using a 1% suspension in bouillon, was added to such a medium inoculated with a hæmolytic streptococcus and incubated for 18 hours, no hæmolysin production was detected with concentrations of 0·24% and 0·48%, and much diminished hæmolysin production in a concentration of 0·12%. The results are set out in Table I. Two strains of streptococci were used, "A" and "Gould," "A" being a hæmolytic streptococcus and "Gould" a non-hæmolytic streptococcus.

TABLE I.

		Hæmolysis.		Growth.	
		Strep. "A."	Strep. "Gould."	Strep. "A."	Strep. "Gould."
1% peptone bouillon containing	0·02% lecithin	++++	−	Good growth	Good growth
" "	0·06% "	++++	−	"	"
" "	0·12% "	+	−	"	"
" "	0·24% "	−	−	"	"
" "	0·48% "	−	−	"	"
1% peptone bouillon (control)		++++	−	"	"

++++, complete hæmolysis; +, trace of hæmolysis; −, no hæmolysis.

That this effect of lecithin on hæmolysin was probably a neutralization of the hæmolysin was shown by growing a hæmolytic streptococcus in peptone bouillon, demonstrating the presence of hæmolysin in the culture, and then adding the same concentrations of lecithin which gave no hæmolysis in Table I

and leaving on the bench for 2 hours. No hæmolysis could be detected after this period on adding 0·1 c.c. to 1 c.c. of 3% washed ox red blood-corpuscles and incubating for 2 hours. In 20% serum bouillon cultures the streptococcus of course gave much more powerful hæmolysin. The addition of more lecithin was generally necessary before neutralization could be obtained. To 1 c.c. of a serum broth culture of a hæmolytic streptococcus were added varying amounts of a 2% suspension of lecithin in bouillon and the mixtures allowed to stand on the bench for 2 hours. Varying amounts of these mixtures were added to 1 c.c. of blood-cells. The results of adding varying amounts of lecithin to a serum broth culture of the streptococcus are shown in Table II.

TABLE II.

	0·2 c.c.	0·1 c.c.	0·05 c.c.	0·025 c.c.
Culture	++++	++++	++++	++
1 c.c. culture + 0·5 c.c. 2% lecithin	++++	++++	+++	+
„ „ + 0·1 c.c. „	++++	+++	++	+
„ „ + 0·3 c.c. „	++	−	−	−
„ „ + 0·5 c.c. „	+	−	−	−
„ „ + 0·75 c.c. „	+	−	−	−
„ „ + 1·0 c.c. „	−	−	−	−

++++, complete hæmolysis; +++, almost complete hæmolysis; ++, partial hæmolysis; +, trace of hæmolysis; −, no hæmolysis.

Neutralization of the hæmolytic action was also effected by the addition of lecithin to filtrates of cultures. These results support the suggestion by Meader and Robinson (1920) that lecithin added to a medium has an inhibitory effect on the production of streptococcal hæmotoxin.

Hellens (1911) showed that ether extraction of serum bouillon cultures containing streptococcal hæmolysin led to the removal of the hæmolysin in the ether fraction from which it could be obtained on evaporation of the ether. We have been unable to confirm these observations except in so far as the ether-extracted culture was inactivated, no hæmolysin being obtained on evaporation of the ethereal extract. This led us to employ ligroin (B.P. 40°–60°) as an inert solvent, which, whilst it removed lecithin easily, did not inactivate the hæmolysin. It is well established that lecithin is a constituent of blood-corpuscles. Hæmolytic action may then be due to some action the hæmolysin exerts on the lecithin of the blood-corpuscles, although by reason of the work of Ponder and McLachlan (1927) the proteins are probably the more important factors. It was therefore deemed necessary to investigate this lecithin-hæmolysin mixture. Serum bouillon cultures containing streptococcal hæmolysin were shaken three times with two to three times their volume of ligroin. The ligroin fraction was run off into a 50 c.c. Claisen flask and the ligroin removed at 30°–36° C. under slightly reduced pressure, the serum bouillon being similarly treated. In every experiment it was found that the hæmolysin was unaffected by treatment with ligroin. Serum bouillon cultures containing streptococcal hæmolysin neutralized with lecithin were treated in an analogous manner. Treatment with ligroin did not disturb the neutralization of the hæmolytic power by the lecithin, *i.e.* the cultures were still non-hæmolytic after extraction, nor on removal of the ligroin from the ligroin fraction was there any trace of hæmolytic action to be observed.

Summary.

1. Lecithin added to cultures of hæmolytic streptococci neutralizes the hæmolytic activity.

2. Treatment with ligroin does not remove or destroy hæmolysin.

3. The hæmolytic activity of streptococcal cultures cannot be restored by treatment with a lecithin solvent (ligroin) after it has once been destroyed by addition of lecithin.

We should like to express our indebtedness to the Medical Research Council for a grant in aid.

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SKIN REGENERATION AND CANCER.

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DOES repeated scarification of an area of the skin result in aberration of the process of repair, and lead to the induction of cancer? Further, is an area of skin which has been the site of repeated injury and repair more susceptible to the carcinogenetic action of tar than the normal skin? The experiments described in this paper were carried out in the attempt to solve these problems.

PRELIMINARY EXPERIMENTS ON THE REPAIR OF INJURY TO THE SKIN OF THE MOUSE.

Preliminary experiments were carried out to determine a suitable method of bringing about a localized injury to a small area of the skin of the mouse, and to investigate the process of repair. The skin on the back of the neck of 20 mice was epilated with sodium bisulphide. The mice were then divided into two batches. The epilated area of the skin of one half was lightly scarified with a needle. The scarification of the other half was carried out by lightly rubbing with sand-paper so as to remove the epidermis over a small part of the skin. Mice from each set were killed at intervals of 1 hour,

2 hours, 6 hours and then every 24 hours. Frozen sections were cut of the injured area. Examination of the sections showed that by lightly rubbing with sand-paper it was possible to bring about a more uniform superficial lesion than by scarifying with a needle. The former method was therefore employed in the subsequent experiments.

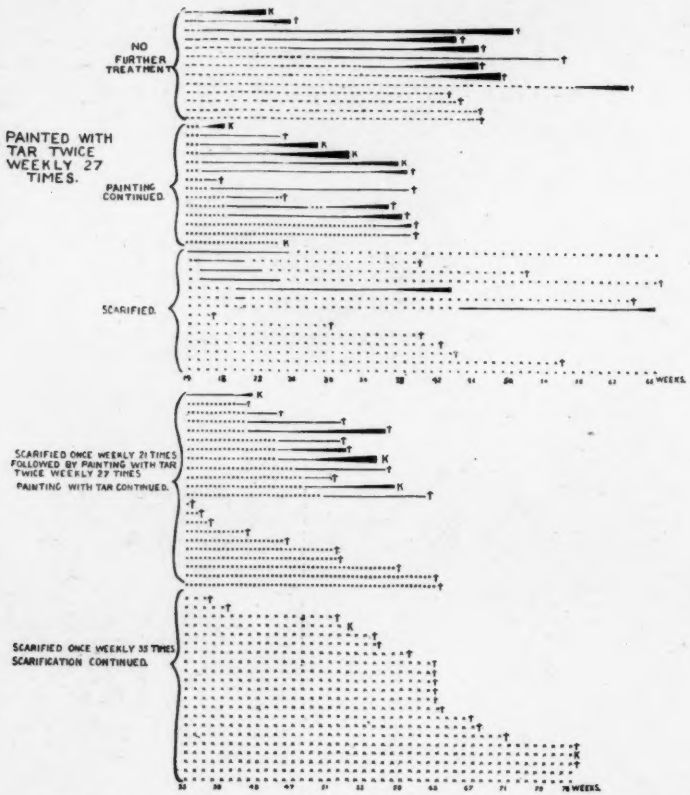
NO FURTHER TREATMENT
PAINTED WITH TAR TWICE WEEKLY 27 TIMES.
PAINTING CONTINUED.
SCARIFIED.
SCARIFIED ONCE WEEKLY 21 TIMES FOLLOWED BY PAINTING WITH TAR TWICE WEEKLY 27 TIMES
PAINTING WITH TAR CONTINUED.
SCARIFIED ONCE WEEKLY 35 TIMES
SCARIFICATION CONTINUED.
WEEKS.

FIG. 1.—Chart showing the general plan of the experiments and the results. (Explanation in the text.)

The changes occurring in the epidermis after scarification may be summarized briefly as follows:

(1) The epidermal cells immediately surrounding the lesion, and also those of the torn hair-follicles, begin to spread out, and to migrate over the exposed dermis beneath the superficial coagulated exudate. The behaviour of these cells is strikingly similar to that of the cells of a fragment of tissue cultivated *in vitro*.

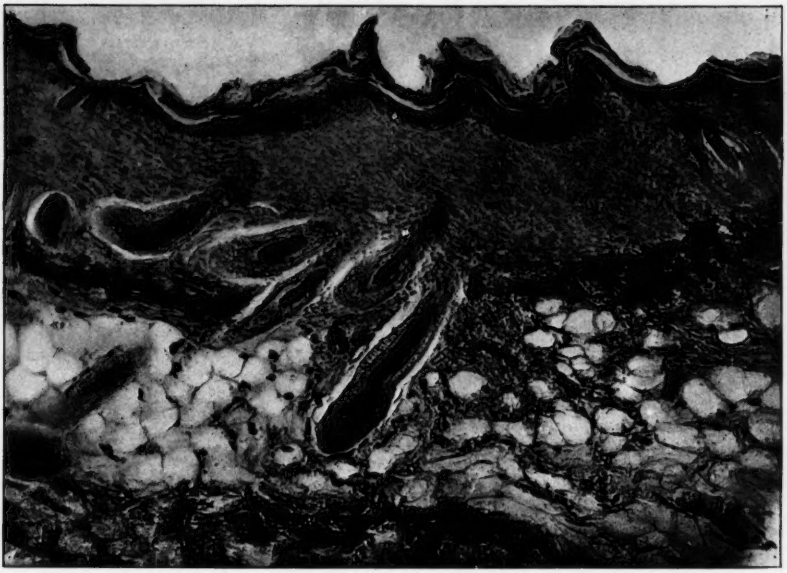

FIG. 2.—Transverse section of the area of the skin of a mouse which has been scarified once weekly for a year, showing the area of sclerosis displacing the elastic fibres.

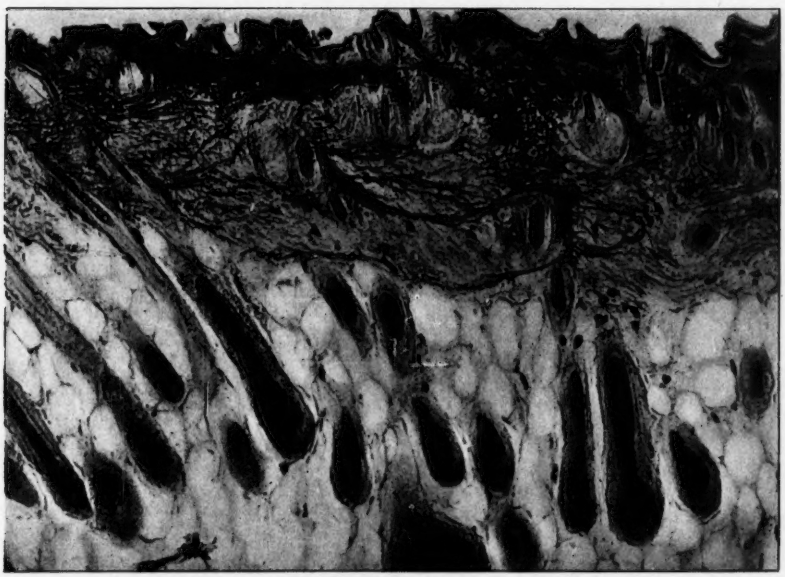

FIG. 3.—Transverse section of the normal skin of the same mouse for comparison with Fig. 2. The elastic fibres run right up to the epidermis.

Ludford.

(2) Cells around the margin of the lesion, and also in the hair-follicles, show numerous mitotic figures. The cells thus formed spread over the injured area until the epidermal covering is replaced.

(3) The newly formed layer of epidermal cells increases considerably in thickness by cell-division, until ultimately it is thicker than the normal epidermis. The superficial cells undergo keratinization and form a surface layer of keratin, and repair of the injury is completed.

The time taken for the completion of this process varies according to the size of the area of the skin scarified, and the extent of the injury to the dermis. In the following experiments the amount of scarification performed was such that the process of repair was completed and the skin restored to the normal condition within a week.

THE EFFECT OF SCARIFYING AN AREA OF SKIN PREVIOUSLY PAINTED WITH TAR.

Fifty mice were painted on the back of the neck with tar twice weekly over a period of 13 weeks. During this time 9 died; the remaining 41 were divided into three sets. Thirteen received no further paintings; 14 others were painted as before; the remaining 14 were scarified once weekly on the area of skin previously painted with tar. The scarification was performed as described above by rubbing off the epidermis with sand-paper.

The results of this experiment are shown diagrammatically in the chart, Fig. 1. Progressively growing carcinomata are indicated by thickened black lines. Their rate of growth is suggested by increasing thickness of the lines. These tumours on histological examination show penetration of the panniculus carnosus. Papillomata are represented by continuous lines. Each dot indicates an application of tar, while each cross denotes scarification has been performed. The broken lines of the first group on the chart mean that there has been no painting or scarification. Death of a mouse is shown by a cross at the end of the line. When the animal was killed the letter "K" appears instead. Each line on the chart thus indicates for each mouse the treatment it has received, and whether papilloma or carcinoma developed as the result of that treatment.

Examination of the chart therefore shows that of the mice which received 27 paintings with tar and had then no further treatment, 8 ultimately developed carcinomata, and one that died had papillomata on the painted area. Of those that were painted continuously the same number also developed carcinoma, but in a shorter time. Five mice that died had papillomata on the painted skin.

That tumours may appear after the cessation of painting with tar was first shown by Leitch (1922), while Findlay (1925) found that a single application of hot tar to the skin of the mouse might be sufficient to induce tumour-formation in rare cases.

Of the 14 mice which were painted with tar 27 times and then scarified on the same area of the skin once weekly, only 2 developed carcinomata, while papillomata appeared on 5 others, but ultimately disappeared. The fact that only 2 of these 14 mice developed cancer can probably be attributed to the scarification rubbing away the epidermal cells which had been subjected to the

action of the tar, although there is the further possibility that the sclerosis which results from scarification may be an important factor.

THE EFFECT OF REPEATED SCARIFICATION, AND OF TAR-PAINTING AFTER SCARIFICATION.

Fifty mice were scarified once weekly, as already described, 21 times. During this time 7 deaths occurred. The remaining 43 mice were divided into two groups. Twenty-one were scarified continuously week by week. The other 22 were painted with tar twice weekly on the area previously scarified. The results of this experiment are shown on the lower half of the chart, Fig. 1. Of the mice which were scarified alone none developed even papillomata. Examination of sections of the skin after scarification weekly, for 78 weeks, showed the epidermis comparatively uniform in thickness. Figs. 2 and 3 are photomicrographs of frozen sections of the skin of a mouse which had been scarified once weekly for a year. Fig. 2 shows a section through the skin of the scarified area; Fig. 3 a section through the skin a slight distance from the former. The epidermis in both is normal in appearance. Fig. 2 shows, however, beneath the epidermis a thickened area consisting largely of fibroblasts and collagen, which has displaced the elastic fibres seen in Fig. 3 running right up to the epidermis.

Of the 22 mice which were scarified 21 times and then painted with tar on the same area of the skin, 5 ultimately developed cancer and 7 papillomata. On comparing this group on the chart with the group of mice which were painted with tar continuously, it will be seen that the initial scarification is responsible for a slight retardation in the development of cancer. This is probably due to the sclerosis caused by scarifying.

While these experiments differ from those of Deelman (1927), they lend no support to his view that injury may have a direct influence on tumour genesis in an area of epithelial cells that are susceptible to tumour-formation as the result of tar-painting.

Conclusions.

(1) Repeated scarification of an area of the skin of the mouse does not result in an aberration of the process of repair, nor lead to the induction of cancer.

(2) An area of the skin which has been the site of repeated injury and repair is not more susceptible to the carcinogenetic action of tar than the normal skin.

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TETANUS.—IX. THE OXIDATION-REDUCTION POTENTIAL OF THE SUBCUTANEOUS TISSUE FLUID OF THE GUINEA-PIG; ITS EFFECT ON INFECTION.

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In a previous paper (Fildes, 1927) it was contended that the failure of tetanus spores to germinate when inoculated alone into guinea-pigs was due to the tension of oxygen in the subcutaneous tissues being too high to permit germination. In another paper (Fildes, 1929) it was shown that tetanus spores in culture did not germinate when the Eh in their surroundings was more positive than + 0·01 volts (pH 7·0).

In the present paper is produced evidence that the Eh of the subcutaneous tissues of the normal guinea-pig is more positive than + 0·01 volts, and it is held that this fact is sufficient to account for the failure of tetanus spores to germinate when occupying this position without introducing as explanation any such concept as phagocytosis.

MEASUREMENT OF THE Eh OF THE SUBCUTANEOUS TISSUE FLUID AND OF THE PERITONEAL FLUID OF THE GUINEA-PIG.

The general idea was to inoculate the animals with indicator dyes in the reduced state and observe whether oxidation took place.

For reduction of the dye the apparatus shown in Fig. 10 of the previous paper (Fildes, 1929) was used. The concentration of the dye was about 0·01%.

Experiment 1.—Two white guinea-pigs were taken and their bellies shaved. One was killed and placed into the incubator for 20 minutes. Each was then inoculated with methylene blue as follows, great care being taken to inject as superficially as possible:

Site 1. 1 c.c. dye solution not reduced.
„ 2. 1 c.c. „ „ reduced.
„ 3. 1 c.c. saline.

The immediate effect as seen through the skin was as follows:

Living guinea-pig.

Site 1. Strong blue colour.
„ 2. Definite „ „
„ 3. *Nil.*

* Working under grants from the Medical Research Council.

Dead guinea-pig.

Site 1. Strong blue colour.
„ 2. No colour.
„ 3. *Nil.*

The object of site 3 was to compare with site 2, the skin of the dead guinea-pig being bluish in colour.

Very rapidly the colour in site 2 in the living guinea-pig approximated to that in site 1. Subsequently up to 60 minutes later, the appearance at all sites remained the same.

The living guinea-pig was now killed and the sites were viewed from the peritoneal surface: both sites 1 and 2 were strongly blue; when cut into they were found strongly blue. In the dead guinea-pig, however, site 2 was colourless, but became strongly blue on treatment with potassium ferricyanide. Site 1 was blue, but the intensity of colour increased a little with potassium ferricyanide.

From this experiment it was concluded that a living guinea-pig's subcutaneous tissues maintained an Eh on the positive side of that of reduced methylene blue, and that this Eh became rapidly more negative on death, presumably because of a fall in the tension of O_2 resulting from cessation of the circulation.

Precisely the same conclusions can be drawn when, instead of methylene blue, either thionin or indophenol 1 (1 naphthol–2 sodium sulphonate indo 2, 6 dibromophenol) is injected.

The following data were obtained with the latter dye:

Experiment 2.—Four guinea-pigs, 2 dead for 20 minutes; all injected in 3 sites as before and the dead animals returned to the incubator.

Guinea-pig 1; living.

	Oxidized.	Reduced.
Immediate	Blue	? Slight blue.
After 10 minutes	„	Fairly definite blue.
„ 20 „	„	Obvious blue.
„ 40 „	„	As blue as oxidized.
Killed and examined	Intense blue	Intense blue.

Guinea-pig 2; living.

Immediate	Blue	? No colour.
After 5 minutes	„	Slight blue.
„ 10 „	„	Definite „
„ 20 „	„	Nearly as blue as oxidized.
Killed and examined	Intense blue	Strong blue, but a little bluer with ferricyanide.

Guinea-pig 3 ; dead.

Immediate	Blue	No colour.
After 60 minutes	,, fading	,,
,, 75 ,,	? No colour	,,
Examined	Slight blue, becoming intense with ferricyanide	,, but becoming intense blue with ferricyanide.

Guinea-pig 4 ; dead.

Immediate	Blue	No colour.
After 50 minutes	,, fading	,,
,, 75 ,,	No colour	,,
Examined	,, but blue with ferricyanide	,, but blue with ferricyanide.

From this it was concluded that the Eh of the subcutaneous tissues of a living guinea-pig was positive to that of reduced indophenol 1. The rate of oxidation was, however, slower than in the case of thionin or methylene blue, because the difference between the Eh of the tissues and the reduction point of the dye was less.

Proceeding now to the next positive dye indicator, indophenol 2 (phenol-indol 2, 6 dibromophenol), the following results were obtained:

Experiment 3.—Technique as before; 3 guinea-pigs; 2 living, 1 dead.

Guinea-pig 1 ; living.

	Oxidized.	Reduced.
Immediate	Blue	No colour.
After 20 minutes	Largely faded	,,
,, 30 ,,	No colour	,,
Killed and examined	,,	,,

Slight return of colour with ferricyanide.

Guinea-pig 2 ; living.

Immediate	Blue	No colour.
After 30 minutes	,, fading	,,
,, 60 ,,	,, ,,	,,
,, 80 ,,	Slight blue	,,
Killed and examined	Blue	,,

No return or increase of colour with ferricyanide.

Guinea-pig 3 ; dead.

Immediate	Blue	No colour.
After 30 minutes	Nearly faded	,,
,, 60 ,,	No colour	,,
Examined	,,	,,

Strong blue with ferricyanide.

It will be observed that with this dye the results are complicated by a decomposition of the dye in the living animal, though apparently not in the dead. This is shown by the failure of potassium ferricyanide to bring back the full colour of the reduced dye. Any deduction is therefore apt to be fallacious.

Experiments were carried out by injecting the animals in different sites at intervals of 15 minutes with reduced dye. The animals were killed and examined 15 minutes after the last injection. No blue was found anywhere, and only in the last site could any be recovered with ferricyanide.

There is thus no evidence of oxidation of this dye by the subcutaneous tissues; but on the other hand it is probable that decomposition had occurred before sufficient time had elapsed for oxidation to take place.

The general conclusion regarding the Eh of the subcutaneous tissue fluids must be, that it is decidedly positive to the zone of complete reduction of indophenol 1. If the oxidation of the reduced dye is of the order of 50%, then the Eh would fall on the negative side of complete reduction of indophenol 2, *i.e.* at pH 7·0* at about Eh + 0·12 volts. On the other hand, *B. tetani* requires at pH 7·0 an Eh + 0·01 volts for germination.

The Eh of the peritoneal fluid was determined with indophenol 1 only, since indophenol 2 decomposed as in the subcutaneous tissues.

The reduced dye was injected, and the animal's peritoneum tapped with a hard glass pipette after 30 minutes. The colour of the issuing fluid was noted. This was always a strong blue, but it became somewhat bluer on addition of potassium ferricyanide. The Eh of this fluid must therefore have been more negative than that of reduced indophenol 2. It should be noted that if any blood escapes into the peritoneal cavity during this experiment, the dye is entirely decomposed and cannot be recovered with potassium ferricyanide.

THE EFFECT OF INJECTIONS OF VARIOUS SUBSTANCES UPON THE Eh OF THE TISSUE FLUIDS.

Having produced experimental support for the suggestion that the natural immunity to infection by *B. tetani* depends upon the too positive Eh of the tissues, it remains to examine how the essential facts relating to infection fit in with this hypothesis.

In a previous paper (Fildes, 1927) it was shown that circumscribed areas of necrosis, whether produced by $CaCl_2$, sterile earth or aleuronate, were necessary features of germination under the condition of the experiment. It was supposed that these areas, by causing capillary thrombosis or otherwise, prevented the maintenance of the normal oxygen tension, at least during the initial stages of the lesion before organization set in. This would lead to "anaerobic conditions" in the area involved in the same way as ligation of the vessels of the testicle.

It is now necessary to establish this view by showing that such areas are at an abnormally negative oxidation-reduction potential.

* There is little agreement upon the pH of tissue fluids, but it is usually considered to be in the region of pH 7·0. According to a series of tests carried out for me by Dr. F. Williams with indicator dyes the pH of the guinea pig's subcutaneous and peritoneal fluids falls between pH 6·8 and pH 7·0.

Experiment 4.—Guinea-pig inoculated intramuscularly in the right thigh with 1 c.c. of a mixture consisting of 4 c.c. 5% $CaCl_2$ + 1 c.c. 0·05% methylene blue; into the left thigh with 1 c.c. of a mixture of 4 c.c. saline and 1 c.c. 0·05% methylene blue.

Killed in 24 hours; the sites of inoculation exposed and examined. In the $CaCl_2$ site there was seen a central whitish area about 0·5 cm. in diameter surrounded by a zone of blue. The central white area became blue rapidly on exposure to air. In the other leg a slight blue was found.

This shows that the centre of a $CaCl_2$ lesion has an Eh equal to or more negative than that of completely reduced methylene blue.

Experiment 5.—A guinea-pig was inoculated with 1 c.c. aleuronate mixture intraperitoneally, and 24 hours later 1·5 c.c. of 0·5% methylene blue intraperitoneally. It was killed and examined 3½ hours later. On opening the peritoneal cavity, free methylene blue was found and a patchy staining of the intestines and peritoneal wall. Numerous small aleuronate abscesses were seen. These were all colourless, but became blue under observation. When the belly was again closed, these abscesses bleached and again became blue on exposure to air. The methylene blue in the peritoneal cavity was diffusing through the wall of the abscess into the abscess fluid, where it became reduced.

It thus follows that the fluid in an aleuronate abscess has an Eh on the negative side of that of reduced methylene blue.

THE QUESTION OF INTRACELLULAR Eh.

The whole of the work previously described has had reference to the fate of spores lying in the extracellular tissue fluids as governed by the Eh in these fluids; it will, however, be noted that the Eh of these fluids, as determined by the behaviour of dyes in them, must differ from the Eh of some other parts of the body, since dyes on intravenous injection are well known to be reduced. It may therefore be objected that tetanus spores when intravenously injected should germinate in those parts of the body where reduction of the dyes takes place. This, however, is not the case.

The reason for this seems to be clear. When spores are injected intravenously they either remain in the extracellular tissue fluids or are phagocytosed. When dye is injected intravenously it does not remain in the extracellular spaces, but is taken up by certain cells and reduced.

Experiment 6.—Rabbit injected intravenously with 5 c.c. 2% methylene blue in saline. Rapid general blueing followed by bleaching. Killed in 5 minutes and rapidly examined:

Heart blood.

(1) Diluted with saline = no blue.
(2) + ferricyanide = no blue.

Kidney Cortex Excised.

(1) Becomes blue on exposure.
(2) Fragment dropped into saline shows no diffusion of blue.
(3) The saline pipetted off + ferricyanide = no blue.
(4) The fragment treated with ferricyanide = strong blue (especially after boiling).

From this it was concluded that the dye had been removed from the circulation by the kidney-cells, and that it was actually intracellular and not in the extracellular fluids.

Thus the fact of reduction of methylene blue after intravenous injection does not imply that there are areas of extracellular fluid which are at a negative oxidation-reduction potential suitable for the germination of spores.

On the other hand, the experiment does show that the interior of kidney-cells at least has great reducing intensity, and it may be suggested that if the same Eh is present in the interior of polymorphonuclear cells, the phagocytosed spore should germinate. This is never the case.

It is not at present possible to dispose of this objection. The only information relating to the Eh of the interior of cells has been supplied by Needham and Needham (1925–1926), working for the most part with marine eggs. These they found by micro-injection methods to have an intracellular Eh very similar to the Eh of the subcutaneous fluids as described in this paper. If the Eh of the polymorphonuclear cell is the same, this would account for the lack of germination. However, there is no information on this point, and in any case the objection is hardly valid. It has been shown clearly (Fildes, 1929) that Eh is not the sole factor in germination, and it is obvious that germination cannot be expected under conditions which lead to rapid solution of the ingested non-sporing bacillus.

DISCUSSION.

It seems clear from the present experiments that an essential condition for germination of tetanus spores *in vivo* is the same as *in vitro*, namely, an adequately negative state of oxidation-reduction potential. In nature it may be supposed that this generally results from a slight suppuration about an infected foreign body. Though, however, oxidation-reduction potential may be the main factor, other factors no doubt also come in, for instance, pH. This may operate directly on the spores or indirectly by means of its effect upon Eh. In a previous paper (Fildes, 1929) it was shown that tetanus spores hardly germinated at all on the acid side of pH 6·3; thus, if the bacteria causing the suppuration had the effect of depressing the pH below this, germination would not occur in spite of the adequate Eh. On the other hand, an alkali-producing organism might have the same effect as alkali in a culture, and lead to a more rapid negative change in Eh and thus more certain germination. It will be recalled that *B. prodigiosus* was formerly a favourite means for "activating" spores, and that this, in culture, induces a high pH. Considerations of this sort may account for the anomalous behaviour of different bacteria used in these experiments.

There seems little reason to doubt that the main conclusions relating to *B. tetani* may be applied in principle to all anaerobic organisms.

When deprived of products of previous growth they cannot maintain active existence in normal tissues because of the incompatible Eh in these tissues. It may be pointed out that all infections due to an acknowledged anaerobe are mixed; the anaerobe is not developing in normal tissues, but in fluids which have been already affected by another organism. This is not only the case

with the sporing anaerobes, but also in such diseases as acne or actinomycosis. It may be assumed that the associated organism causes the necessary depression of oxidation-reduction potential.

This necessary depression would not, however, be equal with all anaerobes, since some are more anaerobic than others. According to a few experiments carried out along the lines already described, *B. welchii* will germinate at an Eh positive to or about the zone of complete reduction of indophenol 1. Future work will probably show that the critical Eh of other bacteria is to be found in zones increasingly positive until that of the normal tissue fluids is approached.

The more positive of them may grow without the necessity for any special measures to induce anaerobiosis, but they will still be anaerobes so far as the normal tissues are concerned and will not grow in these normal tissues. The nearer, however, the critical Eh of a bacterium approaches that of the tissues the more readily will the bacterium find conditions favourable to growth.

Considerations of this sort may account for the varying behaviour of anaerobes in the tissues. It may be surmised that in the case of bacteria which require (or prefer) an Eh negative to that of the tissue fluids, infection in the sense of effective local multiplication can only take place when the local Eh has been adequately reduced by other bacteria, trauma or other conditions. When the Eh of the bacterium is only slightly below that of the tissues the degree of mixed infection or trauma need only be slight.

Infection having taken place, the subsequent spread of the bacteria may depend chiefly upon the effect that their own products have upon the Eh of the tissue fluids. It will be recalled that the lysins (? aggressins) of several bacteria are in themselves the reduced form of some compound which is inactive when oxidized (Neill, 1925; Neill and Mallory, 1926). Thus they are reducing agents, and would facilitate the forward spread of the bacteria which had secreted them if these bacteria required (or preferred) an Eh more negative than that of the normal tissue fluids.

Proceeding with this line of argument, it seems possible that Eh enters into some of the pathological relations of strictly aerobic organisms.

It is possible to conceive of the normal tissues being insusceptible to infection because their Eh is too negative, or at least to ascribe the variations in pathological processes to fluctuations in the Eh requirements of tissues and bacterium. The failure of tubercle bacilli to propagate in the interior of caseous foci may be recalled, or the probable depression of local Eh produced by surgical collapse of the lung and its effect in retarding the progress of the disease; indeed even "rest" may be supposed to diminish the circulation, lower the oxygen tension in the tissue fluids, and so make conditions less satisfactory for any tubercle bacilli situated there.

SUMMARY AND CONCLUSIONS.

1. The Eh of the subcutaneous tissue fluids and of the peritoneal fluid of the normal guinea-pig is more positive than the Eh required for germination of spores of *B. tetani*.

2. To this fact chiefly is ascribed the failure of tetanus spores to germinate when inoculated into these animals.

3. The germination of spores which takes place naturally in tetanus or

which is experimentally induced is due to the production in the tissues of an adequately negative Eh.

4. The relation of oxidation-reduction potential to infection and pathological processes is discussed.

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THE OXYGEN REQUIREMENTS OF *B. PESTIS* AND PASTEURELLA STRAINS.

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AN unexpected difficulty in obtaining the development of colonies of *B. pestis* on agar plates was the occasion of the experiments detailed here. What so frequently happened was that, while the first plate of a dilution series would be so well grown as to be covered with confluent growth, the second plate would remain absolutely sterile. One received the impression that thinly sown plates were unfavourable for growth and that plague bacilli lying widely separated from one another on an agar surface were incapable of multiplication. In an endeavour to supply to such isolated microbes the stimulus which thickly sown plates seemed to possess, small quantities of broth culture of *B. pestis* sterilized by heat were added to the agar with which the plates were poured. The development of colonies on such augmented plates was a normal one; the inhibition of growth, previously noted on ordinary agar, no longer occurred.

A quantitative estimation of the augmented colonial growth thus achieved was obtained by seeding diluted broth cultures of *B. pestis* into agar with and without the addition of sterilized broth culture of the same organism. The result is shown in Experiment 1.

Experiment 1.

Five capillary drops of 1/10,000 broth dilution of a 1-day broth culture of—	Colonies in agar plates.	
	Without augmentor.	With 0·3 c.c. of *B. pestis* broth culture sterilized at 60° C. for 1 hour.
B. pestis . .	0	1409

Here, as elsewhere, incubation is at 37° C. and lasts 5 days before the plates are counted. The volume of agar per plate was 10 c.c.

The difference between the two plates is obvious. The augmenting effect is obtainable with 1-day cultures but is more pronounced when they are 8 to 10 days old; it is obtainable with filtrates as well as with whole broth culture; it is obtainable with many kinds of bacteria besides *B. pestis*, but not with all. To this point further reference is made later.

This stimulation of growth is not due to any alteration of pH in the medium as the addition of such small amounts of broth culture makes no appreciable alteration in that respect; neither is it due to the so-called V factor necessary for the growth of *B. influenzæ* which is present in broth cultures of bacteria as well as in vegetable tissues, for experiments carried out with fresh potato extract which contains this V factor, proved its inability to initiate growth in thinly sown plates of *B. pestis*.

That the phenomenon was one associated with oxygen tension was suggested by a comparison of the growth resulting from the sowing of a dilute plague culture into an agar shake with that from a similar inoculation on an agar plate. While the plate remained sterile, the agar shake showed a rich development of colonies extending to within ¾ inch of the surface, the tube remaining free of colonies to that depth. It was also possible to induce growth in thinly sown agar plates by the addition of a reducing agent such as sodium sulphite or merely by placing them under anaërobic conditions (see Experiment 2).

Experiment 2.

Five capillary drops of 1/10,000 broth dilution of 24-hour broth culture of—	Colonies in agar plates.			
	Without augmentor.	With augmentor.	With 0·025% sodium sulphite.	At 1 mm. atmospheric pressure.
B. pestis	0	350	313	298

There is evidence, therefore, that for the initiation of growth of plague bacilli an oxygen tension lower than that obtaining in ordinary agar plates is necessary.

After the completion of these experiments similar observations by Drennan and Teague (1917) were brought to our attention; they were confirmed later by Meyer and Batchelder (1926). Drennan and Teague had also found a difficulty in the colonial culture of *B. pestis*. This they ascribed to the presence of inhibitory substances in the agar medium consequent on its autoclaving or even its heating in the steamer. Finding that the addition of sodium sulphite encouraged growth, they regarded this substance as having a neutralizing effect upon the supposed inhibitory substances but did not consider the possibility of its action as a reducing agent. In the same way they considered that the development of otherwise inhibited plague colonies which they observed taking place in the neighbourhood of a variety of organisms growing on the agar plates, was due to the production by these adjuvant bacteria either of similar neutralizing substances or of vitamin-like bodies, which so stimulate growth that inhibition no longer occurs.

The work of Webster (1925) and Webster and Baudisch (1925) deals with

the oxygen tension requirements of another bacterium. A very similar sensitivity has been demonstrated here in respect of *P. lepiseptica*. It was found that seedings into broth of as many as 20,000 per c.c. did not result in growth unless the oxygen tension was lowered by (1) occlusion of the air by a layer of vaseline, (2) the addition of a reducing substance such as blood or certain iron salts, viz. Fe_2O_3 and Fe_3O_4. Webster also found that this sensitivity of *P. lepiseptica* was confined to the smooth or D variant of De Kruif (1921) which grows with diffuse turbidity in broth and that his rough or G variant which produces a granular turbidity in broth grew as well in broth open to the air as in broth layered with vaseline or to which blood has been added.

In the following experiments the sensitivity of pasteurellas to oxygen tension has been investigated alongside that of *B. pestis*.

It may be mentioned here that a number of other organisms, including *B. pseudo-tuberculosis rodentium*, *B. tuberculosis*, *B. dysenteriae* of Shiga and Flexner, have been tested for oxygen sensitivity but in no case was the oxygen tension as exerted in aërobic agar plates found to inhibit growth.

In Experiment 3 both *B. pestis* and a pasteurella were employed and the effects compared of the addition of blood in the form of a peptonized solution, as advocated by Fildes (1920) for the growth of *B. influenzae*, and of sterile broth culture. It will be seen that both substances are capable of inducing colonial growth which, without them, is inhibited.

Experiment 3.

Five capillary drops of broth dilution of a 1-day broth culture of—	Colonies in agar plates.		
	Without augmentor.	With 0·3 c.c. sterile broth culture.	With 0·3 c.c. peptonized blood solution.
B. pestis (1/10,000 dilution)	9	544	512
P. aviseptica (1/100,000 dilution)	0	272	304

In Experiment 4 the relative sensitivity of three pasteurella strains is compared. *R50G* represents the G variant of De Kruif which, according to Webster, is oxygen insensitive, *R50D* and *121* are diffusely growing D variants. It will be seen that the G variant is, as Webster states, very largely insensitive to oxygen and grows almost as well without as with augmentors. Smooth variants, however, are not all equally oxygen sensitive; while 560 viable cells of *P. aviseptica 121* are entirely suppressed for lack of a lowered oxygen tension, of 456 viable cells of *P. leptiseptica R50D*, 104 developed into colonies in spite of that lack. This variation in the oxygen sensitivity of apparently quite smooth cultures has been noticed in other pasteurella strains.

Experiment 4.

Five capillary drops of 1/100,000 broth dilution of a 1-day broth culture of—	Colonies in agar plates.	
	Without augmentor	With 0·3 c.c. peptonized blood
P. lepiseptica R50G (rough)	135	177
P. lepiseptica R50D (smooth)	104	456
P. aviseptica 121 (smooth)	0	560

It remained to be seen whether this insensitivity of the rough pasteurella variant might not be due to the fact that the bacteria growing in a granular fashion in broth are sown in clumps which possibly retain traces of the augmenting substance in their interstices.

Agar cultures of *Lepiseptica R50G* and *Lepiseptica 1387*, both rough variants, were accordingly emulsified in distilled water and diluted in the same fluid, thus giving suspensions which were seen to consist of isolated unclumped organisms. Experiment 5 demonstrates that even under this condition insensitivity to oxygen is maintained.

Experiment 5.

Five capillary drops of 1/100,000 distilled water dilution of a 1-day broth culture of—	Colonies in agar plates.	
	Without augmentor	With 0·3 c.c. sterile broth culture.
P. lepiseptica R50G (rough)	146	188
P. lepiseptica 1387 (rough)	223	325

Webster found that blood maintained its augmenting power after subjection to heat. This is not the case with the broth cultures used for augmentation.

In Experiment 6 a broth culture of *B. paratyphosus B* previously sterilized for 1 hour at 60° C. was divided and portions heated for 5 minutes at 70°, 80°, 90°, and 100° C. respectively; peptonized blood was employed unheated and heated for 5 minutes at 100° C.

Experiment 6.

Five capillary drops of broth dilution of a 1-day broth culture of—	Colonies in agar plates.							
	Without augmentor.	With augmentor, unheated or heated for 5 minutes at various temperatures; 0·3 c.c. per plate.						
		S.B.C.	S.B.C. (70°).	S.B.C. (80°).	S B.C. (90°).	S.B.C. (100°).	P.B.	P.B. (100°).
B. pestis (1/10,000 dil.)	15	784	592	544	1	0	793	780
P. aviseptica (1/100,000 dil.)	0	272	55	20	0	0	304	192

S.B.C. = sterilized broth culture; P.B. = peptonized blood solution; figures in brackets = degree to which these were heated for five minutes.

It is seen that while peptonized blood remains active even after subjection to 100° C. for 5 minutes, broth culture loses its power of initiating growth when heated to temperatures higher than 70° C.

To ascertain whether the destruction is a purely thermal one and not an oxidation of some reducing substance taking place rapidly at higher temperatures, the heating of the broth culture was now carried out *in vacuo*. The test-tube containing 1·5 c.c. of augmentor was washed out with nitrogen after each of three evacuations which were carried out to 1 mm. of mercury pressure. The result as seen in Experiment 7 was the same.

Experiment 7.

Five capillary drops of broth dilution of a 1-day broth culture of—	Colonies in agar plates.				
	Without augmentor.	With 0·3 c.c. of sterile broth culture heated for 5 minutes to—			
		80° C.	80° C. *in vacuo.*	100° C.	100° C. *in vacuo.*
B. pestis (1/10,000 dilution)	0	187	166	0	0
P. suiseptica (1/100,000 dilution)	0	270	250	0	0

The substance in broth cultures which so lowers oxygen tension in an agar plate as to permit of the growth of isolated cells of either *B. pestis* or pasteurella is, therefore, a thermolabile one and its rapid destruction in the absence of oxygen at temperatures between 70° and 80° C. suggests that it is of enzymic nature.

A very striking parallel exists between the production of this enzyme and that of the catalase investigated by M'Leod and Gordon (1923). According to these workers bacteria fall into two large groups, the members of one producing catalase, the members of the other not.

The catalase non-producers are the anaerobes, streptococci, pneumococci and Shiga bacilli which are, in fact, also non-producers of augmenting substance. Similarly the members of the catalase-producing group correspond almost entirely with those of the augmentor-producing group, the chief exceptions being the gonococci and meningococci; only 1 out of 4 meningococcus strains yielded broth cultures with augmenting powers, neither of 2 gonococcus strains did, although M'Leod and Gordon regarded these organisms as particularly strong catalase-producers. It is possible that their feeble growth in liquid media affords an explanation of the discrepancy; certainly the only broth culture that showed heavy growth was the one which possessed augmentor.

The following lists comprise the organisms tested for augmenting powers after growing in broth in 6 days and sterilizing at 60° for 1 hour, the figures in brackets representing the number of strains tested:

Microbes producing augmentor: *Paratyphosus B* (2), *Suipestifer* (2), *Morgan I* (1), *Coli* (1), *Flexner* (3), *Typhosus* (3), *Lactis aerogenes* (1), *Acidi lactici* (1), *Prodigiosus* (2), *Staphylococcus aureus* (2), *Staphylococcus albus* (2), *Subtilis* (1), *Mesentericus* (1), *Diphtheroids* (1), *Diphtheriae* (2), and *Meningococcus* (1).

Microbes not producing augmentor: *Anaerobes* (3), *Streptococcus* (5), *Pneumococcus* (2), *Shiga* (3), *Gonococcus* (2), *Meningococcus* (3), *Cholerae* (1), *Melitensis* (1), *Acidi lactici* (4), *Diphtheroids* (4), *Staphylococcus albus* (2), and *Diphtheriae* (2).

It will be noticed that certain inconsistencies exist. Among the strains which were tested of diphtheroids, *C. diphtheriae*, *B. acidi lactici*, meningococcus and staphylococcus, were certain members which did and others which did not produce augmentor. It is probable that if the number of strains examined were increased, similar inconsistencies might be found in other species

as well. The only occasion on which one might ascribe the inconsistency to variation in amount of growth and consequent variation in amount of enzyme present in the culture was in the case of one of the meningococci as already mentioned above. For the rest, the broth cultures seemed all equally well grown.

SUMMARY.

A difficulty in initiating the growth of plague- and pasteurella-cells lying widely scattered on agar plates has been observed and regarded as due to the oxygen sensitivity of those organisms.

This difficulty can be overcome by the addition to the culture medium of such substances as blood, sodium sulphite or sterilized broth culture of a variety of bacteria or by placing the plates under anaerobic conditions.

It is suggested that the substances just mentioned act as reducing agents and that the substances contained in broth cultures of these particular bacteria are enzymic in nature, as they are rapidly destroyed under anaerobic conditions between 70° C. and 80° C.

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THE TRANSMISSIBILITY TO RABBITS OF POLIOMYELITIS VIRUS.

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POLIOMYELITIS can be readily transferred to monkeys with the production of symptoms and lesions analogous to those found in the human disease and once established in the monkey nervous system the virus possesses great virulence for these animals. Such a state of affairs unfortunately has not been found to occur with any regularity so far as other animals are concerned and consequently the monkey has been the experimental animal employed in the investigation of this disease. This naturally has many disadvantages as,

owing to the cost and attention necessary, research work can be performed only on a very restricted scale. In order to overcome this difficulty attention was given to the possibility of utilizing young rabbits for such work, as several authors have claimed that they have produced a modified form of the disease in these animals.

Krause and Meinicke (1909, 1910) claimed to have reproduced the disease in rabbits by inoculation of different tissues, cerebro-spinal fluid, blood, brain and spleen, from a human case. Lentz and Huntemuller (1910) stated they were able to pass the disease to young rabbits, 600–1000 gm. in weight. The histological lesions, however, were very slight compared with those found in monkeys and consisted of hyperæmia with some nerve-cell degeneration. Marks (1911), using young rabbits, propagated the virus through several series and was able to reproduce the typical disease in monkeys by inoculation of brains from the second, fourth and sixth rabbit passages. The rabbits died between the eighth and fifteenth day usually suddenly. At autopsy, hyperæmia of the cerebral cortex only was found and histological examination revealed nothing of a definite nature. Rosenau and Havens (1916) stated that the use of young rabbits, preferably under 6 weeks old, was essential and that following inoculation, usually intracranial, of the virus, two types of infection could be distinguished, (1) a "progressive" form and (2) a "fulminating" form. The average incubation period was 12 days and the duration of the disease varied from 1–7 days. The histological lesions were not typical of the disease in man or monkey. Congestion, punctate hæmorrhages and degeneration of the motor cells were found but perivascular infiltration was absent. In view of this it was considered that the rabbit reacted differently to the virus from man or monkey.

On the other hand, Flexner and Lewis (1910), in spite of repeated attempts with human and monkey strains of virus, were unsuccessful in their efforts to infect rabbits, while Landsteiner and Levaditi (1910) only infected 1 rabbit out of a large number and reviewing the situation they concluded that the rabbit was useless for poliomyelitis research. Romer (1911), using young and old animals, was unable to confirm the previous findings of Kraus and Meinicke and decided that these animals were not receptive to the poliomyelitis virus. Leiner and v. Weisner (1911) employing some 52 rabbits, mainly young, about 6 weeks old, were unable to reproduce the disease in these animals. Amoss (1918) inoculated poliomyelitis virus intracerebrally into rabbits without producing any symptoms and found, by re-inoculating the brain around the site of injection into monkeys, that the virus persisted actively for 4 but not for 7 days. Nevin and Bittman (1924), using some 50 young rabbits were unsuccessful in their efforts to reproduce the condition; in only 2 cases were symptoms comparable to poliomyelitis in man found and then the histological lesions were of a totally different nature—cellular infiltration of the nervous system being absent.

The claim is thus made that under certain ill-defined conditions rabbits have succumbed to inoculations of the poliomyelitis virus after an incubation period generally of some 8–15 days. The histological findings have not been typical of this disease but it has been claimed that a modified form has been produced in these animals, which must, of necessity, be young.

EXPERIMENTAL.

Rabbits, 4–6 weeks old and 400–650 gm. in weight, have been employed throughout this work. The inoculations have been given intracerebrally, intravenously, intraperitoneally and by a combination of these routes. The former has been the one usually adopted. For intracerebral inoculation, ether anæsthesia was utilized and a hole, through which the virus was injected, bored through the cranium by means of manual pressure on an ordinary upholstery tack. The marginal vein of the ear was used for the intravenous method and the virus given as a filtrate.

(a) *Monkey strain of virus.*

Three such strains have been employed, all virulent for monkeys, two especially so. 5% emulsions in physiological saline, which have proved successful on intracerebral inoculation into monkeys, were generally prepared although on a few occasions emulsions were given of 10% and 25% strength. In this group are included those animals, which received the inoculations by any of the above-mentioned routes. The results are shown in Table I.

TABLE I.

Route.	Number of rabbits.	Result.	
		Died.	Survived.
(1) Intracerebral	18	6	12
(2) Intravenous	3	...	3
(3) Intraperitoneal	2	...	2
(4) 1 + 3	1	...	1
Total	24	6	18

It will be seen that 6 animals only, all intracerebrally inoculated, succumbed. 4 of the 6 died within 24 hours of the operation, death being probably the immediate result of the anæsthetic or operation—shock or hæmorrhage and not to the direct specific action of the poliomyelitis virus. Of the other two, both died suddenly one after 7 days and the other after 17. Unfortunately, an autopsy was performed only on the former when no obvious lesions were found in the central nervous system or in any of the other tissues to account for death. Histological examination was also negative. Subsequent intracerebral passages of a 10% emulsion of the brain of this animal, after 4 months preservation in 50% glycerine at a temperature of 4° C., into three rabbits, proved uneventful. The histological examinations were performed by my colleague, Dr. E. W. Hurst.

It was conceivable that the virus, while not giving rise to any obvious symptoms or signs of reaction in the animal, might have in some way sensitized the central nervous system so making it more responsive to further doses of the virus. Consequently, the intracerebral inoculations on 14 occasions were repeated at a later date but again with negative results. Some time after the

inoculations many of the animals were etherized and examined histologically but no lesions of an outstanding nature were discovered.

The presence of some form of reaction occurring in the animals following the virus inoculations was sought by daily examinations of their rectal temperatures and body weights. It was found that for a few days immediately after the operation the weight either remained stationary or decreased slightly, to be then followed by a steady gain; nothing markedly irregular was noted in the temperature readings.

(b) *Human virus.*

As the use of poliomyelitic virus from monkey tissues did not prove very fruitful, a human strain was employed. This seemed rational, as it is postulated that the human strain undergoes some modification on monkey passage whereby the virulence for these animals is greatly augmented. If such be the case the human strain might possess a greater affinity for the rabbit than the monkey virus. Unfortunately, while the virus employed was obtained from a typical case of poliomylitis, it was found unable to reproduce the disease in monkeys, owing probably to some decrease in virulence during transit, although it must be borne in mind that the initial transfer of the virus from human nervous tissue to the monkey is only successful in approximately 50% of attempts. The negative results obtained are therefore of doubtful value. They are given in Table II.

TABLE II.

Route.	Number of rabbits.	Result.	
		Died.	Survived.
Intracerebral	4	...	4
Intracerebral + Intraperitoneal	2	...	2
Total	6	...	6

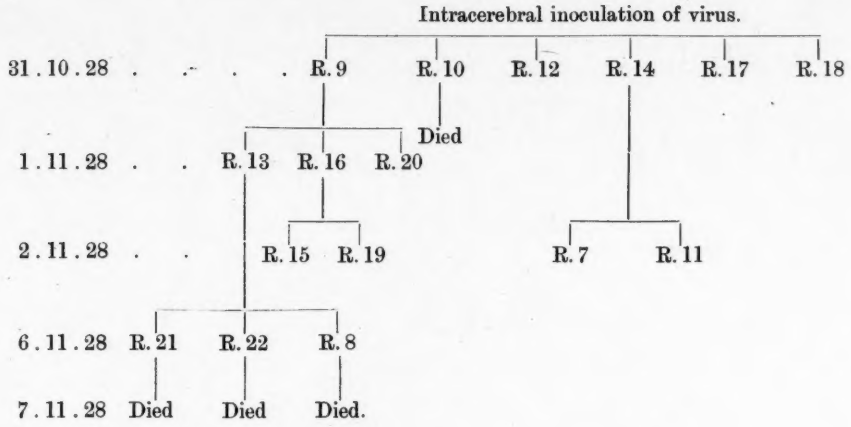
The animals remained quite well and about a month later three were again inoculated intracerebrally. Except that one rabbit died shortly after the inoculation nothing of a definite nature was observed.

(c) *Accommodation experiments.*

Human and monkey strains of the virus yielded, therefore, negative results when inoculated by various routes into young rabbits. The next point to receive consideration was the possibility of reproducing the disease in these animals by accommodating or adapting the virus to their tissues.

The virus emulsion was inoculated intracerebrally into 6 young rabbits (R. 23–28). One of these (R. 26) was etherized in 24 hours and the region of the brain round the site of inoculation removed, emulsified and inoculated intracerebrally into 3 more rabbits (R. 29–31). All the animals remained well except R. 27, which died of cerebral hæmorrhage 4 days after inoculation.

The following is the scheme of another experiment performed on similar but more extensive lines:


Intracerebral inoculation of virus.
31.10.28 . . . . R.9 R.10 R.12 R.14 R.17 R.18
Died
1.11.28 . . R.13 R.16 R.20
2.11.28 . . R.15 R.19 R.7 R.11
6.11.28 R.21 R.22 R.8
7.11.28 Died Died Died.

All animals survived except those shown. These, with the exception of R. 10, died of a pasteurella infection.

Amoss, as already stated, has demonstrated the presence of poliomyelitis virus in the rabbit brain-tissue around the site of inoculation 4 days at least after intracerebral inoculation of the virus in rabbits. Thus it seems justifiable to assume that the virus would have been present in the brains of rabbits R. 9 and R. 14 removed 24 hours and 48 hours respectively after the original inoculation and also in the brain of R. 16, which was inoculated with the brain of R. 9. Again, nothing to suggest the occurrence of poliomyelitis even in a modified form was observed.

DISCUSSION.

Evidence, clinical or histological, of the reproduction of poliomyelitis in young rabbits has not been obtained from the experiments performed in this work. Three animals succumbed following the virus inoculations in 6, 7 and 17 days respectively without having exhibited any appreciable symptoms and on that score could be placed in the "fulminating rabbit poliomyelitis" category of Rosenau and Havens. Other findings, however, rule out at least one of the three. The rabbit dying on the 6th day was found to have a generalized pasteurella infection and so could be excluded from this group. Of the other two, one was not autopsied; the other exhibited no definite histological lesions and later passage of the brain yielded negative results. It might be argued that 4 months in 50% glycerine at 4° C. might have caused the virus to deteriorate—this is possible but against it is the fact that human and monkey strains can be preserved for long periods (8 years according to Rhoads, 1929) by such means. Again, rabbits are susceptible to spontaneous encephalitis and the possibility of the presence of this disease has always to be considered in evaluating the results of any experiments in which the central nervous system of these animals is involved.

CONCLUSIONS.

The results obtained give no support to the belief that young rabbits react even in a modified form to the virus of poliomyelitis. They cannot therefore be recommended as experimental animals for the investigation of this disease.

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NOTES ON INFECTIOUS MYXOMATOSIS OF RABBITS.

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Infectious myxomatosis was first described by Sanarelli (1898) as the result of an outbreak among rabbits in the animal house of the Institute of Hygiene in Monte Video. The disease is restricted to certain countries of South America—the Argentine, Brazil and Uruguay—and is highly pathogenic for rabbits, although the hare of South America (*Lepus brasiliensis*) can also be infected with difficulty. No other animals are known to be susceptible. These facts are of interest from the palæo-pathological point of view for, according to Scott (1924), species of the genus Lepus only appeared as migrants in South America at the end of the pliocene period. The virus must therefore have appeared or at least adopted a parasitic existence at what, geologically speaking, is a comparatively recent date.

Material.

Three strains of the virus myxomatosum were available. One was obtained directly from Prof. H. de Beaurepaire-Aragao, of Rio de Janeiro, the others

from Prof. Sanarelli, in Rome, through the kindness of Dr. Lipschütz, of Vienna. My thanks are due to all these gentlemen. One of the strains sent by Prof. Sanarelli was that used by Dr. Rivers at the Rockefeller Institute, New York. The material was received in glycerine.

Clinical Symptoms.

The disease produced by all the three strains was identical. The material, finely minced and suspended in physiological saline was either injected subcutaneously into the eyelids or rubbed into the scarified skin. Four to five days later the site of inoculation was swollen; the swelling gradually increased to about the size of a walnut while other swellings appeared at the root of the ears, in the tissues of the scrotum, prepuce and around the mouth and anus. In the female, the orifice of the vagina was swollen. A muco-purulent discharge came from the nose and conjunctivæ. With the appearance of a swelling at the site of inoculation there was a gradual loss of appetite with consequent emaciation. Death took place in from seven to twelve days. The disease may also be transmitted by intravenvous or intraperitoneal injection and by instillation into the conjunctival sac or into the nares. Intravenous inoculation of the virus is followed in from seven to ten days by the appearance of swellings in the eyelids and in the scrotum.

Moses (1911) showed that when injected intravenously the virus can be localized in any area of skin by plucking out the hairs. This finding was confirmed. In addition it was shown that a similar localization in any desired area can be brought about by the repeated injection of histamine, a phenomenon which has been demonstrated in the case of other viruses and bacteria (Findlay, 1928).

The disease is extremely infectious and even in a large airy room it is difficult to avoid infecting other rabbits.

The virus could not be transmitted to rats, mice, guinea-pigs, pigeons or chickens. Through the kindness of Dr. E. Hindle inoculations were made in a *Macacus rhesus* monkey and in striped Chinese hamsters—*Cricetulus griseus*—with negative results. A Belgian hare, however, was found to be susceptible. A suspension of the virus in physiological saline was active in dilutions of 1/1,000,000. The virus passed through Berkefeld N and Chamberland L_1 filters with a reduction of potency to 1/10,000. It was retained by Chamberland L_2 candles. The virus survives in 50% glycerine for at least four months, while tissues containing the virus can be dried in a dessicator and kept at room temperature for at least a month without losing their virulence. When suspended in physiological saline and kept at 37° C. the virus was still alive after six days.

In addition to the material obtained from the swollen nodules the conjunctival secretion, the blood and an emulsion of liver were all found to be infective.

Pathological Changes.

On sectioning the tumour-like masses they appeared pinkish in colour and slimy to the touch. There was a curious smell resembling that of carrion. There was no enlargement of the lymph-glands or spleen, but a considerable

degree of emaciation was invariably present. The following fixatives were used: formol saline, Zenker's fluid, with and without acetic acid, and Regaud's solution. Sections were stained with Heidenhain's iron hæmatoxylin and van Gieson, eosin and methylene blue, Giemsa, toluidin blue and van Gieson, Gram's stain and Weigerts' elastica stain. Schridde's method was used for the demonstration of mitochondria, the Mann-Kopsch technique for the Golgi apparatus, and the Feulgen technique for thymo-nucleic acid.

Histologically the chief feature of the nodular swelling is the enormous dilatation of the connective-tissue spaces which are filled with a mucinous deposit. This mucinous degeneration involves the connective tissues both of the cutis vera and subcutaneous tissues. Scattered throughout the swollen tissues are connective-tissue cells, many of which are enormously hypertrophied. There is no apparent increase in the number of the connective-tissue cells and even in early stages of the lesions it is by no means easy to discover even a single connective-tissue cell in mitotic division. The nodular lesions are thus due, not to an active proliferation of the tissue elements as in the Rous sarcoma or fowl pox, but simply to the myxomatous changes in the tissues.

When secondary infection occurs the swollen tissues contain a large number of polymorphonuclear leucocytes and occasional eosinophils. The capillaries of the affected tissue are dilated, the capillary endothelium swollen, while here and there are small extravasations of blood.

The epidermal cells show little or no gross change, except when secondary infection occurs when the whole of the epidermis may be slough. Nucleolar extrusions were found as in any normal epidermis, while occasionally some of the cells of the basal layer had a ballooned appearance. In sections of the swellings stained with osmic acid a large number of fatty globules were seen, partly extracellular, partly within the enlarged connective-tissue cells. The testicles, apart from congestion, were normal even when the scrotum was affected. The internal organs were congested and on making blood cultures a short Gram negative bacillus was occasionally isolated.

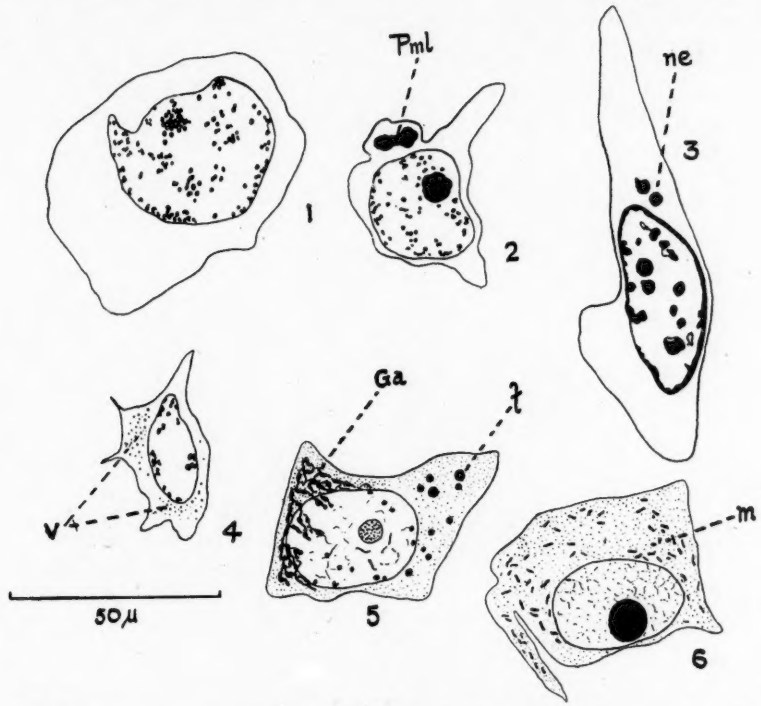
Virus Inclusions.

As in so many other virus diseases a variety of intracellular inclusions have been ascribed to the presence of the virus. Splendore (1909) described, after Giemsa staining, both in the connective-tissue cells and occasionally in the leucocytes inclusions resembling the trachoma bodies of Halberstadter and von Prowazek.

Moses (1911) failed to confirm the presence of these trachoma-like bodies. Aragao (1911) found in the nuclei of the hypertrophied connective-tissue cells a rarification of the chromatin, together with small round elementary bodies to which he gave the name *Chlamydozoön myxomæ*. Rivers (1926) emphasized the presence of changes in the epidermal cells. The epidermal cells first became enlarged, then small pink granular areas appeared in the cytoplasm, increasing rapidly in size and involving most of the cytoplasm. In the centre of the acidophilic masses blue round or rod-shaped bodies were often

seen. Finally there were distinct vesicles in the epidermis not unlike those in herpes simplex.

Aragao (1927) believes that certain fine granules, which he states have a diameter of 0·1 μ, represent the virus. These rounded bodies, which he terms *Strongyloplasma myxomæ* may be demonstrated by staining with Loeffler or Giemsa after making very fine smears from fresh tumours, previously expressed



The figures represent hypertrophied connective-tissue cells from myxomatous lesions in the rabbit.

Fig. 1.—The chromatin is broken up to form small granular masses; the nucleolus has disappeared; fixed Zenker's fluid; stained Heidenhain's iron hæmatoxylin and van Gieson.

Fig. 2.—The nucleolus is still intact; a polymorphonuclear leucocyte has been phagocytosed (*pml*); fixed Zenker's fluid; stained Heidenhain's iron hæmatoxylin and van Gieson.

Fig. 3.—Nucleolar extrusions (*ne*); fixed Zenker's fluid; stained Heidenhain's iron hæmatoxylin and van Gieson.

Fig. 4.—Minute granules (*v*) in the cytoplasm, possibly representing the virus; fixed Zenker's fluid; stained iron hæmatoxylin and van Gieson.

Fig. 5.—Hypertrophied Golgi apparatus (*Ga*) and fat-globules (*f*). Mann-Kopsch method.

Fig. 6.—Rod-shaped mitochondria (*m*); fixed Schridde's method; stained Heidenhain's iron hæmatoxylin.

between two sheets of filter-paper, or by filtering the virus through a film of agar which is subsequently removed by washing. Lipschütz (1927) described

in the cytoplasm of the connective-tissue cells small bodies either alone or in little masses, varying in size from a just visible granule to a large coccus. Larger forms, diplococcal or even angular in shape, were also described. In addition to the myxoma cells, similar granules were found in the much swollen connective-tissue cells of the deeper layers of the skin and also in the endothelial cells lining the lymph spaces. Lipschütz gave these granules the name of *Sanarellia cuniculi.*

There is thus a considerable difference of opinion as to which, if any, of the bodies described actually constitute the virus. In the present investigation it has been quite impossible to determine the presence of any inclusions in the epidermal cells. This is the more surprising as one of the strains is said to be the same as that used by Rivers (1926). The following possibilities suggest themselves:

1. The infective material used by Rivers contained in addition to the virus myxomatosum another virus which is not preserved by glycerine.

2. The rabbits used by Rivers harboured another virus which was stirred into activity by inoculation of the virus myxomatosum.

Intranuclear changes similar to those described by Aragao (1911) are shown in Figs. 1 and 2 where the chromatin network has been entirely split up to form small irregular masses. The nucleolus may have entirely disappeared or may still be distinct as in Fig. 2. These small masses stain by the Feulgen technique showing that they are composed of thymo-nucleic acid. Fig. 3 shows the presence of nucleolar extrusions in an hypertrophied connective-tissue cell. Fig. 4 stained by Giemsa's method shows the presence of numerous very minute red stainining granules scattered throughout the cytoplasm. These granules appear to correspond with the granules described by Aragao (1927) and Lipschütz (1927). Similar granules may be seen in some of the endothelial cells lining the capillaries and lymph spaces; they are not stained by the Feulgen technique. These granules are quite distinct from the mitochondria which, as shown in Fig. 6, are definitely rod-shaped, nor are they associated with the hypertrophied Golgi apparatus seen in Fig 5. In the present state of our knowledge it is of course impossible to state definitely whether these minute granules actually constitute the virus, though from their uniform distribution in the cells of the lesions their close relationship may be suspected.

CONCLUSIONS.

The symptoms and pathological changes in infectious myxomatosis of rabbits are described. In the connective-tissue cells of the lesions certain minute granules are present which may possibly represent the actual virus.

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OBSERVATIONS UPON ELECTRIC CHARGE IN CERTAIN BACTERIOLOGICAL PROBLEMS.

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IN a previous communication (Brown and Broom, 1929) a description has been given of the technique of cataphoresis as applied to bacterial suspensions, using the type of cell devised by the authors. In the following experiments this cell has been used, but a second, modified from that suggested by Northrop (1922), has also been employed.

The general structure of this cell resembles that of Northrop. It, however, differs in that the glass which forms the base is only 0·75 mm. in thickness, which enables dark-ground illumination to be employed with a $\frac{2}{3}$-in. objective and a × 25 eye piece. The other main point of difference is that one-way taps are used above and below each end of the cell, the advantage of this arrangement being that the cell can be filled, and bubbles eliminated, much more easily than with the three-way taps employed in Northrop's cell. The cell is illustrated in Fig. 1, and is so constructed as to allow the taps suitable clearance on each side of the stage of a Zeiss microscope. The necessary strength is obtained by cementing flattened glass rods along the whole length of the top of the cell on either side. It is a technical advantage to drill out the holes in the taps to a diameter of 3 mm.

The cell is filled as follows: With the zinc electrodes in position, the lower compartments are filled with a saturated solution of sulphate of zinc by means of a capillary pipette introduced through both the upper taps. When the canals in the lower taps have been completely filled the pipette is withdrawn, the lower taps closed, and the cell thoroughly flushed out first with tap-water and then with distilled water. The cell proper is then filled in the same

manner with a fresh capillary pipette, and any bubbles which remain can be readily dislodged by applying a rubber teat to one of the upper tubes and exerting slight alternate pressure and suction. Before an observation is made the upper taps are closed and the lower taps opened.

The cell can be rapidly disinfected *in situ* by drawing neutralized formaldehyde through it; this does not affect the bitumen which is used to cement the cell. Scrupulous chemical cleanliness is essential in the technique of cataphoresis, since traces of such salts as thorium nitrate will materially effect the results obtained. Thorium nitrate should be considered as much a contamination as is *B. subtilis* in other bacteriological procedures.

The results with this cell do not materially differ from those obtained with the one previously described. It appears, however, to have certain advantages

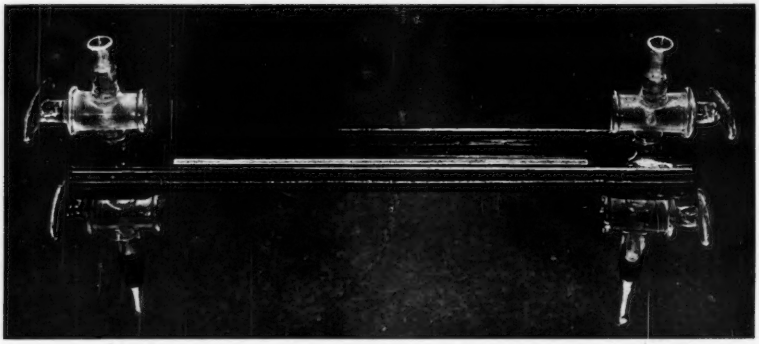

FIG. 1.

in that it is totally closed, in some ways is easier to manipulate, and the potential gradient does not alter in the more concentrated solutions as it does when agar bridges are employed.

CATAPHORESIS EXPERIMENTS WITH ROUGH AND SMOOTH VARIANTS.

From a study of rough and smooth forms of pneumococci, Falk (1928) comes to the conclusion that the progressive conversion of smooth to rough is accompanied by apparently gradual, not discontinuous changes in P.D. (potential difference).

We have been unable to demonstrate any appreciable difference in the P.D. of rough and smooth variants of the coli-typhoid group when washed and suspended in distilled water.

In our experiments we have taken the three criteria, colony formation, salt stability and type of growth in broth as evidence of the rough or smooth form.

Bruce White (1929), having shown certain definite differences in the behaviour of rough and smooth variants to chemical reagents, it appeared possible that similar differences might occur in cataphoretic migration under the influence of solutions of salts of polyvalent metals, such as thorium.

The technique which we have employed was similar to that previously described by us, and the results here also are expressed in terms of μ per second calculated for a potential gradient of 1 volt per cm. Figs. 2 and 3 are typical of the results obtained, and show the migration rates under these conditions of *B. typhosus* Rawlins and *B. aertrycke* Glasgow respectively. It will be noticed that in both cases the migration rate of the rough variant in a total concentration of thorium nitrate of the order $N/10^{-3.5}$ is considerably greater than that of the corresponding smooth form under the same conditions. In a series of several hundred observations on various members of the typhoid, *coli* and salmonella groups, this relationship has been constant in type.

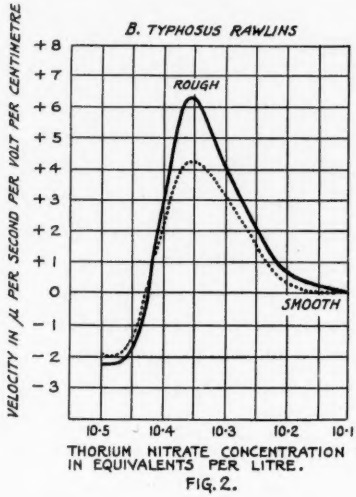

FIG. 2.

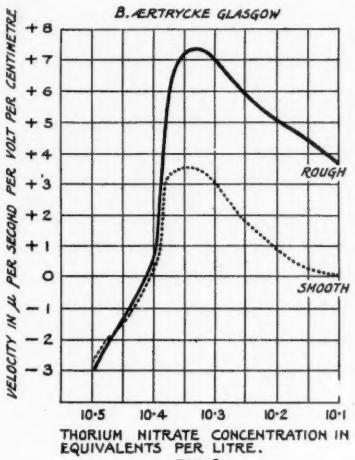

FIG. 3.

THEORETICAL CONSIDERATIONS.

So far we have merely stated the results as they have occurred, and no attempt has been made to explain the mechanism. First, as regards the origin of the potential difference, Northrop (1928), in discussing the nature and origin of the charges of colloidal suspensions, says: "The theory of Helmholtz and Lamb gives us no information as regards the origin of this potential difference, and no satisfactory theory has been suggested up to the present." On the other hand, Winslow, Falk and Caulfeild (1924), came to the conclusion that the migration of bacteria resembled that of protein particles, and their behaviour as regards cataphoresis could be explained on the basis of a Donnan equilibrium.

If these assumptions are correct it would appear that the results which we have obtained are due to differences in the cell membranes of the rough and smooth variants respectively. We are not, however, satisfied that this will explain our results, and further work on the subject is being undertaken.

Electrical Charge in Relation to the Adhesion Phenomenon.

Laveran and Mesnil (1901), studying *T. lewisi* infections in wild rats, observed adherence of leucocytes to the trypanosomes *in vivo*. Ledingham (1908 and 1912) drew attention to the adhesion of cocci and leucocytes when discussing the mechanism of phagocytosis. The phenomenon was subsequently (1917) known as the Rieckenberg reaction, but in view of the former work which had been done on the subject, Davis and Brown (1927) suggested the name of "the adhesion phenomenon." Brown and Davis (1927) showed that adhesion between leptospira and bacteria takes place in presence of a specific immune serum containing complement, whereas no such adhesion occurs when a normal serum is used, and these observers applied this test to the differentiation of leptospira. Levaditi and Mutermilch (1910), working with trypanosomes, came to the conclusion that the phenomenon is of a physico-chemical nature, not demanding vitality on the part of the leucocyte.

It seemed to us possible that alteration in the electric charge carried by the organism under the influence of the immune serum might offer an explanation of this phenomenon.

Reitstötter (1920), in studying the different protein fractions of normal and immune sera, found that certain fractions of immune sera sensitized ferric oxide sols much more strongly than the same fractions of normal sera. This was especially marked with dysentery, diphtheria and tetanus. Northrop and De Kruif (1922) drew attention to the effects of immune serum in lowering the P.D. of bacteria. Shibley (1924), working with pneumococcus and *B. paratyphosus* B, has also shown that the effect of immune serum is to reduce the charge on the organism in a striking way.

To prove whether our conclusions regarding the probable alteration of charge on leptospira by the addition of immune serum is correct, the obvious procedure would be to study the migration rates of leptospira under these conditions. Owing, however, to the rapid movements of this organism in a fluid medium, together with the fact that its vitality is impaired when washed and suspended in distilled water, up to the present we have been unable to obtain accurate measurements of migration rates. The viscosity and complex nature of ordinary media in which leptospira are grown prohibit cataphoresis measurements being made directly on a culture.

Indirect Methods of Studying the Electric Charge in the Adhesion Reaction.

From its general behaviour in a cataphoresis cell we know that leptospira carries a negative charge: the bacteria also are negatively charged, and under ordinary conditions, *e.g.* in the presence of normal serum, they mutually repel each other. When, however, the leptospira are acted upon by a specific immune serum containing complement, their charge is probably altered, with the result that the leptospira become attractive to the bacteria. Now if this reaction is due to electrical attraction between the leptospira and the bacteria, by substituting a negatively charged colloid for the bacteria we should find that the leptospira are more susceptible to its action in the presence of an immune serum.

TABLE I.

					10 minutes.	2 hours.
Normal serum	+	Cu	+	leptospira .	Actively motile .	Actively motile.
Immune „	+	„	+	„ .	All dead .	All dead.
Normal „	+	saline	+	„ .	Actively motile .	Actively motile.
Immune „	+	„	+	„ .	„ „ .	„ „

Table I shows a typical example of the effect of a negatively charged colloid on leptospira in presence of immune and normal sera respectively. In this experiment equal volumes of fresh sera, Oppenheimer's "oscol" colloidal copper, 1 in 2000 Cu and culture of leptospira were allowed to interact, and examined at the times stated. It will here be seen that the colloidal copper is much more toxic to leptospira in the presence of immune serum. Various other negatively charged colloids, viz. gold, sulphur and congo red, have yielded similar results.

There is, therefore, some reason for believing that the adhesion reaction is due to alteration in the electric charge of the organism acted upon by the immune serum. This assumption is strengthened by the result of an experiment in which a positively charged colloid is used in place of the colloidal copper, as shown in Table II. It will be seen that there is no evidence that Victoria blue is more toxic to leptospira in presence of an immune serum than with normal serum.

TABLE II.

							15 minutes.
Normal serum	+	0·1%	Victoria blue	+	leptospira	.	All dead.
Immune „	+	0·1%	„	„	+ „	.	„ „
Normal „	+	0·01%	„	„	+ „	.	Some alive.
Immune „	+	0·01%	„	„	+ „	.	„ „
Normal „	+	0·001%	„	„	+ „	.	All alive.
Immune „	+	0·001%	„	„	+ „	.	„ „

With the 0·01% some were alive 20 hours afterwards both with normal and immune serum.

It might be argued that the toxicity of colloidal copper in presence of fresh immune serum is partially due to the copper and partially due to the lytic action of the immune serum. This, however, does not appear to be so, for in the first place there is a great difference between times to death of leptospira in presence of immune serum and copper, and leptospira with immune serum and saline. In some experiments leptospira exposed to immune serum and saline survived for 20 hours. Further, Schüffner and Mochtar (1927) have shown that the zone of dilution in which lysis takes place is definitely remote from that in which we have been working.

POSSIBLE THERAPEUTIC APPLICATIONS.

Kolmer (1926) states that "Chemo-serotherapy, or the combination of chemotherapy with serum therapy, has been before the profession for several years and is deserving of the closest study and attention."

Although the literature on this subject is scanty, there are several references to the enhanced action of certain drugs in presence of immune serum.

Moore (1915) showed that a single dose of optochin (ethylhydrocupreine), which by itself has practically no protective effect, increased the value of Type II antipneumococcus serum at least 50 times in pneumococcal infections in mice. He emphasizes the fact that a homologous serum must be used.

Various previous observers, Neufeld and Engwer (1912), Engwer (1913) and Boehucke (1913), had also shown a markedly enhanced effect (both protective and curative) when optochin was combined with antipneumococcus serum. The evidence, however, is not clear that strictly homologous serum was used in these cases.

So far as we are aware no adequate theory has as yet been advanced for the explanation of these results with optochin. Taking our results with negatively charged colloids into consideration together with the above, we suggest that the enhanced effect of optochin in presence of homologous immune serum is possibly due to the alteration of electric charge of the organism by the immune serum, which increases the chemotactic effect of negatively charged drugs.

We have examined optochin suspended in freshly boiled distilled water, and find that it does carry a definite negative charge.

If our assumptions are correct there would appear to be new prospects for the possibilities of chemoserotherapy, and investigations on these lines are in progress.

APPLICATIONS IN SERODIAGNOSIS.

Table I shows that a negatively charged colloid is considerably more toxic to leptospira in presence of a specific immune serum than with normal serum. We have obtained similar results with trypanosomes. This reaction can obviously be utilized for the detection of specific immune bodies; for instance, supposing that we are dealing with a wild rat population which it is necessary to examine for the presence of antibodies to *Leptospira icterohæmorrhagiæ*, all that is required is to mix the sera of the rats with a culture of leptospira and a suitable negatively charged colloid. Examinations as regards life or death of the leptospira are made at intervals, controls with normal rat's serum being employed.

Owing to the work of Rieckenberg (1917), Kritschewski and Tscherikower (1925), Krantz (1926), and Messik (1927), the thrombocytobarin or adhesion reaction is well established in trypanosomiasis, spirochætosis and Leishmania tropica infections. It would therefore appear that the employment of negatively charged colloids would offer the possibility of a more definite test for the presence of antibodies than the reaction in its present form.

SUMMARY.

A simple modification of the Northrop microcataphoresis cell is described. Its construction is such that it can be used with dark-ground illumination.

The effect of salts of polyvalent metals on the cataphoretic behaviour of rough and smooth variants of the typhoid-paratyphoid group is described.

The mechanism of the thrombocytobarin or adhesion reaction is considered,

and is correlated with the enhanced toxic action of negatively charged colloids in presence of immune serum.

Certain possible therapeutic and diagnostic applications arising from these observations are suggested.

We wish to express our thanks to Prof. Donnan for his interest and criticisms; also to Dr. St. John-Brooks for kindly supplying us with the cultures used, and to Mr. E. Millwood not only for making the cell but also for working out the various details of its construction.

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ON THE ANTIBACTERIAL ACTION OF CULTURES OF A PENICILLIUM, WITH SPECIAL REFERENCE TO THEIR USE IN THE ISOLATION OF *B. INFLUENZÆ.*

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WHILE working with staphylococcus variants a number of culture-plates were set aside on the laboratory bench and examined from time to time. In the examinations these plates were necessarily exposed to the air and they became contaminated with various micro-organisms. It was noticed that around a large colony of a contaminating mould the staphylococcus colonies became transparent and were obviously undergoing lysis (see Fig. 1).

Subcultures of this mould were made and experiments conducted with a view to ascertaining something of the properties of the bacteriolytic substance which had evidently been formed in the mould culture and which had diffused into the surrounding medium. It was found that broth in which the mould had been grown at room temperature for one or two weeks had acquired marked inhibitory, bactericidal and bacteriolytic properties to many of the more common pathogenic bacteria.

CHARACTERS OF THE MOULD.

The colony appears as a white fluffy mass which rapidly increases in size and after a few days sporulates, the centre becoming dark green and later in old cultures darkens to almost black. In four or five days a bright yellow colour is produced which diffuses into the medium. In certain conditions a reddish colour can be observed in the growth.

In broth the mould grows on the surface as a white fluffy growth changing in a few days to a dark green felted mass. The broth becomes bright yellow and this yellow pigment is not extracted by $CHCl_3$. The reaction of the broth becomes markedly alkaline, the pH varying from 8·5 to 9. Acid is produced in three or four days in glucose and saccharose broth. There is no acid production in 7 days in lactose, mannite or dulcite broth.

Growth is slow at 37°C. and is most rapid about 20°C. No growth is observed under anaerobic conditions.

In its morphology this organism is a penicillium and in all its characters it most closely resembles *P. rubrum.* Biourge (1923) states that he has never found *P. rubrum* in nature and that it is an "animal de laboratoire." This penicillium is not uncommon in the air of the laboratory.

IS THE ANTIBACTERIAL BODY ELABORATED IN CULTURE BY ALL MOULDS?

A number of other moulds were grown in broth at room temperature and the culture fluids were tested for antibacterial substances at various intervals up to one month. The species examined were: *Eidamia viridiscens, Botrytis cineria, Aspergillus fumigatus, Sporotrichum, Cladosporium, Penicillium,* 8 strains. Of these it was found that only one strain of penicillium produced any inhibitory substance, and that one had exactly the same cultural characters as the original one from the contaminated plate.

It is clear, therefore, that the production of this antibacterial substance is not common to all moulds or to all types of penicillium.

In the rest of this article allusion will constantly be made to experiments with filtrates of a broth culture of this mould, so for convenience and to avoid the repetition of the rather cumbersome phrase "Mould broth filtrate," the name "penicillin" will be used. This will denote the filtrate of a broth culture of the particular penicillium with which we are concerned.

METHODS OF EXAMINING CULTURES FOR ANTIBACTERIAL SUBSTANCE.

The simplest method of examining for inhibitory power is to cut a furrow in an agar plate (or a plate of other suitable culture material), and fill this in with a mixture of equal parts of agar and the broth in which the mould has grown. When this has solidified, cultures of various microbes can be streaked at right angles from the furrow to the edge of the plate. The inhibitory substance diffuses very rapidly in the agar, so that in the few hours before the microbes show visible growth it has spread out for a centimetre or more in sufficient concentration to inhibit growth of a sensitive microbe. On further incubation it will be seen that the proximal portion of the culture for perhaps one centimetre becomes transparent, and on examination of this portion of the culture it is found that practically all the microbes are dissolved, indicating that the anti-bacterial substance has continued to diffuse into the agar in sufficient concentration to induce dissolution of the bacteria. This simple method therefore suffices to demonstrate the bacterio-inhibitory and bacteriolytic properties of the mould culture, and also by the extent of the area of inhibition gives some measure of the sensitiveness of the particular microbe tested. Fig. 2 shows the degree of inhibition obtained with various microbes tested in this way.

The inhibitory power can be accurately titrated by making serial dilutions of penicillin in fresh nutrient broth, and then implanting all the tubes with the same volume of a bacterial suspension and incubating them. The inhibition can then readily be seen by noting the opacity of the broth.

For the estimation of the antibacterial power of a mould culture it is unnecessary to filter as the mould grows only slowly at 37° C., and in 24 hours, when the results are read, no growth of mould is perceptible. Staphylococcus is a very suitable microbe on which to test the broth as it is hardy, lives well in culture, grows rapidly, and is very sensitive to penicillin.

The bactericidal power can be tested in the same way except that at intervals measured quantities are explanted so that the number of surviving microbes can be estimated.

PROPERTIES OF THE ANTIBACTERIAL SUBSTANCE.

Effect of heat.—Heating for 1 hour at 56° or 80° C. has no effect on the antibacterial power of penicillin. Boiling for a few minutes hardly affects it (see Table II). Boiling for 1 hour reduces it to less than one quarter its previous strength if the fluid is alkaline, but if it is neutral or very slightly acid then the reduction is much less. Autoclaving for 20 minutes at 115° C. practically destroys it.

Effect of filtration.—Passage through a Seitz filter does not diminish the antibacterial power. This is the best method of obtaining sterile active mould broth.

Solubility.—It is freely soluble in water and weak saline solutions. My colleague, Mr. Ridley, has found that if penicillin is evaporated at a low temperature to a sticky mass the active principle can be completely extracted by absolute alcohol. It is insoluble in ether or chloroform.

Rate of development of inhibitory substance in culture.—A 500 c.c. Erlenmeyer flask containing 200 c.c. of broth was planted with mould spores and incubated at room temperature (10° to 20° C.). The inhibitory power of the broth to staphylococcus was tested at intervals.

After 5 days complete inhibition in 1 in 20 dilution.

„ 6 „ „ „ „ 1 in 40 „
„ 7 „ „ „ „ 1 in 200 „
„ 8 „ „ „ „ 1 in 500 „

Grown at 20° C. the development of the active principle is more rapid and a good sample will completely inhibit staphylococci in a 1 in 500 or 1 in 800 dilution in 6 or 7 days. As the culture ages the antibacterial power falls and may in 14 days at 20° C. have almost disappeared.

The antibacterial power of penicillin falls when it is kept at room temperature. The rate of this fall can be seen from Table I.

TABLE I.—*Effect of Keeping at Room Temperature on the Anti-Staphylococcal Power of Penicillin.*

	Growth of staphylococcus in dilutions of penicillin as under.											
	1/20.	1/40.	1/60.	1/80.	1/100.	1/200.	1/300.	1/400.	1/600.	1/800.	1/1000.	Control.
At time of filtration .	−	−	−	−	−	−	−	−	−	±	++	++
After 4 days . .	−	−	−	−	−	−	−	−	−	±	++	++
„ 7 „ . .	−	−	−	−	−	−	−	±	+	+	++	++
„ 9 „ . .	−	−	−	−	−	−	−	±	+	+	++	++
„ 13 „ . .	−	−	−	−	−	+	+	+	+	+	++	++
„ 15 „ . .	−	±	+	+	+	+	+	+	+	+	++	++

If the reaction of penicillin is altered from its original pH of 9 to a pH of 6·8 it is much more stable.

The small drops of bright yellow fluid which collect on the surface of the mould may have a high antibacterial titre. One specimen of such fluid completely inhibited the growth of staphylococci in a dilution of 1 in 20,000 while the broth in which the mould was growing, tested at the same time, inhibited staphylococcal growth in 1 in 800.

If the mould is grown on solid medium and the felted mass picked off and

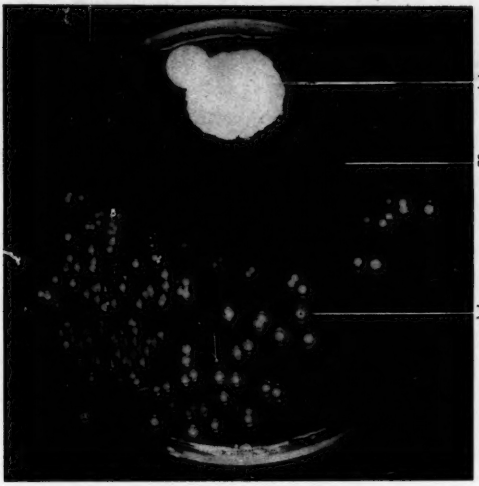

FIG. 1.—Photograph of a culture-plate showing the dissolution of staphylococcal colonies in the neighbourhood of a penicillium colony.

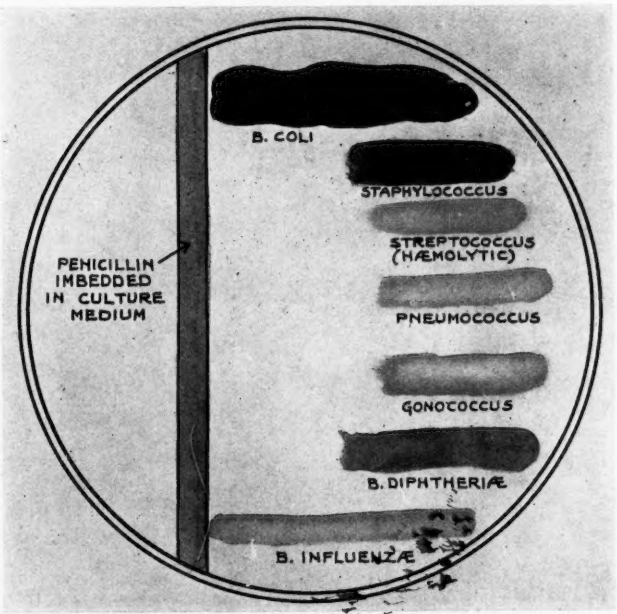

FIG. 2.

Fleming.

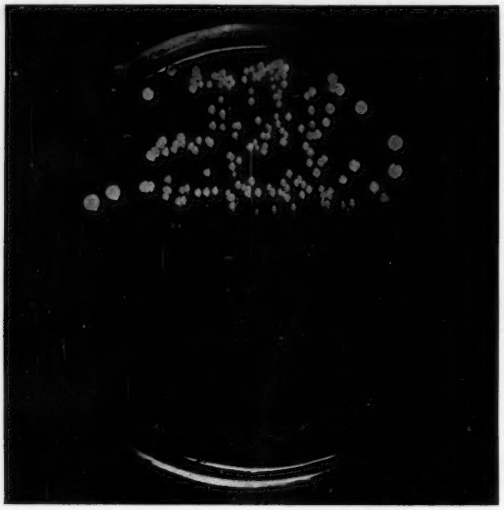

FIG. 3.—Photograph of a culture-plate (Fildes medium) which had been evenly planted with a mixture of staphylococci and *B. influenzæ*. Six drops of penicillin were then spread over the lower half of the plate. Note complete inhibition of staphylococci in the penicillin treated area with resultant pure culture of *B. influenzæ*.

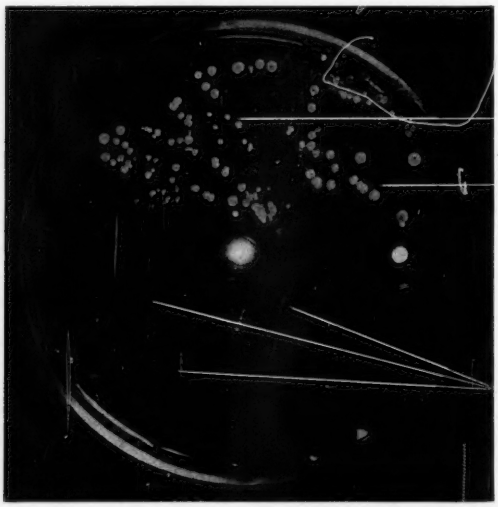

FIG. 4.—Photograph of a culture-plate (Fildes medium) which had been evenly planted with nasal mucus from an individual suffering from a "cold." Six drops of penicillin were spread over the lower half of the plate before incubation. Note profuse growth of staphylococci and diphtheroid bacilli in untreated half, whereas in treated half only some three colonies of *B. influenzæ* are seen.

Fleming.

TABLE II.—*Inhibitory Power of Penicillin (Heated and Unheated) on Various Microbes (Agar Plate Method).*

Type of microbe.	Extent of inhibition in mm. from penicillin embedded in agar, serum agar, or blood agar plates.	
	Unheated.	Boiled for 1 minute.
Experiment 1:		
Staphylococcus pyogenes	23	21
Streptococcus „	17	17
„ *viridans* (mouth)	17	15
Diphtheroid bacillus	27	22
Sarcina	10	10
Micrococcus lysodeikticus	6	7
„ from air (1)	20	16
„ „ (2)	4	9
B. anthracis	0	0
B. typhosus	0	0
Enterococcus	0	0
Experiment 2:		
Staphylococcus pyogenes	24	...
Streptococcus „	30	...
„ *viridans* (mouth)	25	...
Pneumococcus	30	...
Diphtheroid bacillus	35	...
B. pyocyaneus	0	...
B. pneumoniæ (Friedlander)	0	...
B. coli	0	...
B. paratyphosus A	0	...
Experiment 3:		
Staphylococcus pyogenes	16	...
Gonococcus	16	...
Meningococcus	17	...
Experiment 4:		
Staphylococcus pyogenes	17	...
„ *epidermidis*	18	...
Streptococcus pyogenes	15	...
„ *viridans* (fæces)	5	...
B. diphtheriæ (2 strains)	14	...
Diphtheroid bacillus	10	...
Gram-negative coccus from the mouth (1)	12	..
„ „ „ (2)	0	...
B. coli	0	...
B. influenzæ (Pfeiffer) 6 strains	0	..

TABLE III.—*Inhibitory Power of Penicillin on Different Bacteria.*

	Dilution of penicillin in broth.											
	1/5.	1/10.	1/20.	1/40.	1/80.	1/100.	1/200.	1/400.	1/800.	1/1600.	1/3200.	Control.
Staphylococcus aureus	0	0	0	0	0	0	0	0	±	++	++	++
„ *epidermidis*	0	0	0	0	0	0	0	0	±	++	++	++
Pneumococcus	0	0	0	0	0	0	0	0	0	++	++	++
Streptococcus (hæmolytic)	0	0	0	0	0	0	0	0	0	±	++	++
„ *viridans* (mouth)	0	0	0	0	0	0	±	++	++	++	++	++
„ *fæcalis*	++	++	++	++	++	++	++	++	++	++	++	++
B. anthracis	0	0	+	+	++	++	++	++	++	++	++	++
B. pseudo-tuberculosis rodentium	+	+	++	++	++	++	++	++	++	++	++	++
B. pullorum	+	+	++	++	++	++	++	++	++	++	++	++
B. dysenteriæ	+	++	++	++	++	++	++	++	++	++	++	++
B. coli	++	++	++	...	...	...	...	...	...	...	...	++
B. typhosus	++	++	++	...	...	...	...	...	...	...	...	++
B. pyocyaneus	++	++	++	...	...	...	...	...	...	...	...	++
B. proteus	++	++	++	...	...	...	...	...	...	..	...	++
V. choleræ	++	++	++	...	...	...	...	...	...	...	...	++

	1/60.	1/120.	1/300.	1/600.	Control.
B. diphtheriæ (3 strains)	0	±	++	++	++
Streptococcus pyogenes (13 strains)	0	0	0	++	++
„ „ (1 „)	0	0	±	++	++
„ *fæcalis* (11 „)	++	++	++	++	++
„ *viridans* at random from fæces (1 strain)	0	0	0	++	++
„ „ „ „ (2 strains)	0	0	±	++	++
„ „ „ „ (1 strain)	0	±	++	++	++
„ „ „ „ (1 „)	+	++	++	++	++
„ „ „ „ (1 „)	++	++	++	++	++
„ „ at random from mouth (1 „)	0	±	++	++	++
„ „ „ „ (2 strains)	0	0	++	++	++
„ „ „ „ (1 strain)	0	0	0	++	++

0 = no growth; ± = trace of growth; + = poor growth; ++ = normal growth.

extracted in normal salt solution for 24 hours it is found that the extract has bacteriolytic properties.

If this extract is mixed with a thick suspension of staphylococcus suspension and incubated for 2 hours at 45° C. it will be found that the opacity of the suspension has markedly diminished and after 24 hours the previously opaque suspension will have become almost clear.

Influence of the medium on the antibacterial titre of the mould culture.—So far as has been ascertained nutrient broth is the most suitable medium for the production of penicillin. The addition of glucose or saccharose, which are fermented by the mould with the production of acid, delays or prevents the appearance of the antibacterial substance. Dilution of the broth with water delays the formation of the antibacterial substance and diminishes the concentration which is ultimately reached.

INHIBITORY POWER OF PENICILLIN ON THE GROWTH OF BACTERIA.

Tables II and III show the extent to which various microbes, pathogenic and non-pathogenic, are inhibited by penicillin. The first table shows the inhibition by the agar plate method and the second shows the inhibitory power when diluted in nutrient broth.

Certain interesting facts emerge from these Tables. It is clear that penicillin contains bacterio-inhibitory substance which is very active towards some microbes while not affecting others. The members of the coli-typhoid group are unaffected as are other intestinal bacilli such as *B. pyocyaneus*, *B. proteus* and *V. cholerae*. Other bacteria which are insensitive to penicillin are the enterococcus, some of the Gram-negative cocci of the mouth, Friedländer's pneumobacillus, and *B. influenzae* (Pfeiffer), while the action on *B. dysenteriae* (Flexner), and *B. pseudo-tuberculosis rodentium* is almost negligible. The anthrax bacillus is completely inhibited in a 1 in 10 dilution but in this case the inhibitory influence is trifling when compared with the effect on the pyogenic cocci.

It is on the pyogenic cocci and on bacilli of the diphtheria group that the action is most manifest.

Staphylococci are very sensitive, and the inhibitory effect is practically the same on all strains, whatever the colour or type of the staphylococcus.

Streptococcus pyogenes is also very sensitive. There were small differences in the titre with different strains, but it may be said generally that it is slightly more sensitive than staphylococcus.

Pneumococci are equally sensitive with *Streptococcus pyogenes.*

The green streptococci vary very considerably, a few strains being almost unaffected while others are as sensitive as *S. pyogenes.* Gonococci, meningococci, and some of the Gram-negative cocci found in nasal catarrhal conditions are about as sensitive as are staphylococci. Many of the Gram-negative cocci found in the mouth and throat are, however, quite insensitive.

B. diphtheriæ is less affected than staphylococcus but is yet completely inhibited by a 1% dilution of a fair sample of penicillin.

It may be noted here that penicillin, which is strongly inhibitory to many bacteria, does not inhibit the growth of the original penicillium which was used in its preparation.

The Rate of Killing of Staphylococci by Penicillin.

Some bactericidal agents like the hypochlorites are extremely rapid in their action, others like flavine or novarsenobillon are slow. Experiments were made to find into which category penicillin fell.

To 1 c.c. volumes of dilutions in broth of penicillin were added 10 c.mm. volumes of a 1 in 1000 dilution of a staphylococcus broth culture. The tubes were then incubated at 37°C. and at intervals 10 c.mm. volumes were removed and plated with the following result:

	Number of colonies developing after sojourn in penicillin in concentrations as under:				
	Control.	1/80.	1/40.	1/20.	1/10.
Before . . .	27	27	27	27	27
After 2 hours . .	116	73	51	48	23
„ 4½ „ . .	∝	13	1	2	5
„ 8 „ . .	∝	0	0	0	0
„ 12 „ . .	∝	0	0	0	0

It appears, therefore, that penicillin belongs to the group of slow acting antiseptics, and the staphylococci are only completely killed after an interval of over 4½ hours even in a concentration 30 or 40 times stronger than is necessary to inhibit completely the culture in broth. In the weaker concentrations it will be seen that at first there is growth of the staphylococci and only after some hours are the cocci killed off. The same thing can be seen if a series of dilutions of penicillin in broth are heavily infected with staphylococcus and incubated. If the cultures are examined after four hours it may be seen that growth has taken place apparently equally in all the tubes but when examined after being incubated overnight, the tubes containing penicillin in concentrations greater than 1 in 300 or 1 in 400 are perfectly clear while the control tube shows a heavy growth. This is a clear illustration of the bacteriolytic action of penicillin.

TOXICITY OF PENICILLIN.

The toxicity to animals of powerfully antibacterial mould broth filtrates appears to be very low. Twenty c.c. injected intravenously into a rabbit were not more toxic than the same quantity of broth. Half a c.c. injected intraperitoneally into a mouse weighing about 20 gm. induced no toxic symptoms. Constant irrigation of large infected surfaces in man was not accompanied by any toxic symptoms, while irrigation of the human conjunctiva every hour for a day had no irritant effect.

In vitro penicillin which completely inhibits the growth of staphylococci in a dilution of 1 in 600 does not interfere with leucocytic function to a greater extent than does ordinary broth.

USE OF PENICILLIN TO DEMONSTRATE OTHER BACTERIAL INHIBITIONS.

When materials like saliva or sputum are plated it is not uncommon to see, where the implant is thick, an almost pure culture of streptococci and

pneumococci, and where the implant is thinner and the streptococcal colonies are more widely separated, other colonies appear, especially those of Gram-negative cocci. These Gram-negative cocci are inhibited by the streptococci (probably by the peroxide they produce in their growth) and it is only when the mass effect of the streptococci is reduced that they appear in the culture.

Penicillin may be used to give a striking demonstration of this inhibition of bacteria by streptococci and pneumococci. Sputum is spread thickly on a culture plate, and then 5 or 6 drops of penicillin is spread over one half of it. After incubation it may be seen that on the half untreated with penicillin there is a confluent growth of streptococci and pneumococci and nothing else, while on the penicillin-treated half many Gram-negative cocci appear which were inhibited by the streptococci and pneumococci, and can only flourish when these are themselves inhibited by the penicillin.

If some active penicillin is embedded in a streak across an agar plate planted with saliva an interesting growth sometimes results. On the portion most distal from the penicillin there are many streptococci, but these are obscured by coarsely growing cocci, so that the resultant growth is a copious confluent rough mass. These coarse growing cocci are extremely penicillin sensitive and stop growing about 25 mm. from the embedded penicillin. Then there is a zone of about 1 cm. wide of pure streptococci, then they are inhibited by the penicillin, and as soon as that happens Gram-negative cocci appear and grow right up to the embedded penicillin. The three zones of growth produced in this way are very striking.

USE OF PENICILLIN IN THE ISOLATION OF *B. INFLUENZÆ* (PFEIFFER) AND OTHER ORGANISMS.

It sometimes happens that in the human body a pathogenic microbe may be difficult to isolate because it occurs in association with others which grow more profusely and which mask it. If in such a case the first microbe is insensitive to penicillin and the obscuring microbes are sensitive, then by the use of this substance these latter can be inhibited while the former are allowed to develop normally. Such an example occurs in the body, certainly with *B. influenzæ* (Pfeiffer) and probably with Bordet's whooping-cough bacillus and other organisms. Pfeiffer's bacillus, occurring as it does in the respiratory tract, is usually associated with streptococci, pneumococci, staphylococci and Gram-negative cocci. All of these, with the exception of some of the Gram-negative cocci, are highly sensitive to penicillin and by the addition of some of this to the medium they can be completely inhibited while *B. influenzæ* is unaffected. A definite quantity of the penicillin may be incorporated with the molten culture medium before the plates are made, but an easier and very satisfactory method is to spread the infected material, sputum, nasal mucus, etc., on the plate in the usual way and then over one half of the plate spread 2 to 6 drops (according to potency) of the penicillin. This small amount of fluid soaks into the agar and after cultivation for 24 hours it will be found that the half of the plate without the penicillin will show the normal growth while on the penicillin treated half there will be nothing but *B. influenzæ* with Gram-negative cocci and occasionally some other microbe. This makes it infinitely easier to isolate these penicillin-insensitive organisms, and repeatedly

B. influenzæ has been isolated in this way when they have not been seen in films of sputum and when it has not been possible to detect them in plates not treated with penicillin. Of course if this method is adopted then a medium favourable for the growth of *B. influenzæ* must be used, *e. g.* boiled blood agar, as by the repression of the pneumococci and the staphylococci the symbiotic effect of these, so familiar in cultures of sputum on blood agar, is lost and if blood agar alone is used the colonies of *B. influenzæ* may be so minute as to be easily missed.

Figs. 3 and 4 are photographs of culture-plates made after the method described above. On the plate shown in Fig. 3 a mixture of staphylococci and *B. influenzæ* was spread over the whole plate of Fildes medium (Fildes, 1921), then 6 drops of penicillin were spread over the lower half of the plate. The upper half shows the mixed culture while the lower half gives a pure culture of *B. influenzæ*. Fig. 4 represents a culture of nasal mucus from a "cold" made on the same medium. Here, on the upper half (untreated with penicillin) staphylococci and diphtheroid bacilli grow abundantly, while on the treated (lower) half only some three or four colonies of *B. influenzæ* appear.

In conjunction with my colleague, Dr. McLean, a series of cultures were made from the throats of 25 nurses warded for "influenza." The swabs were planted on boiled blood agar and over half of each plate was spread 3 or 4 drops of penicillin. The results are set forth in Table IV.

TABLE IV.—*Summary of Results obtained from Post-Nasal Swabs in 25 Consecutive Cases of "Influenza."*

	Without penicillin.				With penicillin.				Without penicillin.				With penicillin.			
	Pneumococcus or Streptococcus.		*B. influenzæ*.	Gram-negative cocci.	Pneumococcus or Streptococcus.		*B. influenzæ*.	Gram-negative cocci.	Pneumococcus or Streptococcus.		*B. influenzæ*.	Gram-negative cocci.	Pneumococcus or Streptococcus.		*B. influenzæ*.	Gram-negative cocci.
1.	+	+	+	+	−		++	+	14.	+	+	−	−	−	+	−
2.	+	+	++	++	−		++	+	15.	+	+	−	−	−	+	+
3.	+	+	++	+	−		+	−	16.	+	+	−	−	−	+	+
4.	+		−	−	−		+	+	17.	+	+	−	−	−	+	+
5.	+	+	−	−	−		++	−	18.	+	+	−	−	−	+	+
6.	+	+	−	−	−		++	++	19.	+	+	+	−	−	+	++
7.	+	+	−	++	−		+	+	20.	+	+	−	−	−	−	+
8.	+		+	−	−		+	−	21.	+	+	−	−	−	+	+
9.	+	+	−	−	−		+	+	22.	+	+	−	−	−	+	−
10.	+	+	−	−	−		+	−	23.	+		−	+	−	+	+
11.	+	+	−	−	−		+	−	24.	+	+	++	−	−	++	−
12.	+	+	+	++	−		+	+	25.	+	+	−	++	−	−	−
13.	+		++	++	−		+	++								

In the above Table account has only been taken of the common microbes found in these cultures. In some there were a few diphtheroid bacilli which were always penicillin sensitive, and in others there were Gram-negative bacilli which were penicillin insensitive, although they were inhibited by streptococci or pneumococci. Pneumococci and streptococci were classed together, as complete tests were not made to differentiate one from the other.

(From the appearance of the colonies and the morphological characters pneumococci were evidently present in most cases in much larger numbers than were streptococci.)

The swabs were generally planted thickly and in some cases where the growth on the portion of the plate without penicillin was almost confluent, the cultures were sampled by taking smears from thick portions of the growth. In these cases it is possible that the results given do not give a quite complete picture of the cultures. This, however, does not affect the present argument that by the addition of penicillin to the culture medium, and the consequent inhibition of the pyogenic cocci, the isolation of *B. influenzæ* is very much easier. And in a number of cases it was isolated when it was completely missed in the cultures without penicillin.

It is quite immaterial how many pneumococci and streptococci are present in a specimen—they are completely inhibited—and even a few *B. influenzæ* can be isolated from a mixture with an enormous number of these cocci.

From a number of observations which have been made on sputum, post-nasal and throat swabs it seems likely that by the use of penicillin, organisms of the *B. influenzæ* group will be isolated from a great variety of pathological conditions as well as from individuals who are apparently healthy.

DISCUSSION.

It has been demonstrated that a species of penicillium produces in culture a very powerful antibacterial substance which affects different bacteria in different degrees. Speaking generally it may be said that the least sensitive bacteria are the Gram-negative bacilli, and the most susceptible are the pyogenic cocci. Inhibitory substances have been described in old cultures of many organisms; generally the inhibition is more or less specific to the microbe which has been used for the culture, and the inhibitory substances are seldom strong enough to withstand even slight dilution with fresh nutrient material. Penicillin is not inhibitory to the original penicillium used in its preparation.

Emmerich and other workers have shown that old cultures of *B. pyocyaneus* acquire a marked bacteriolytic power. The bacteriolytic agent, pyocyanase, possesses properties similar to penicillin in that its heat resistance is the same and it exists in the filtrate of a fluid culture. It resembles penicillin also in that it acts only on certain microbes. It differs however in being relatively extremely weak in its action and in acting on quite different types of bacteria. The bacilli of anthrax, diphtheria, cholera and typhoid are those most sensitive to pyocyanase, while the pyogenic cocci are unaffected, but the percentages of pyocyaneus filtrate necessary for the inhibition of these organisms was 40, 33, 40 and 60 respectively (Bocchia, 1909). This degree of inhibition is hardly comparable with 0·2% or less of penicillin which is necessary to completely inhibit the pyogenic cocci or the 1% necessary for *B. diphtheriæ*.

Penicillin, in regard to infections with sensitive microbes, appears to have some advantages over the well-known chemical antiseptics. A good sample will completely inhibit staphylococci, *Streptococcus pyogenes* and pneumococcus in a dilution of 1 in 800. It is therefore a more powerful inhibitory agent than

is carbolic acid and it can be applied to an infected surface undiluted as it is non-irritant and non-toxic. If applied, therefore, on a dressing, it will still be effective even when diluted 800 times which is more than can be said of the chemical antiseptics in use. Experiments in connection with its value in the treatment of pyogenic infections are in progress.

In addition to its possible use in the treatment of bacterial infections penicillin is certainly useful to the bacteriologist for its power of inhibiting unwanted microbes in bacterial cultures so that penicillin insensitive bacteria can readily be isolated. A notable instance of this is the very easy isolation of Pfeiffers bacillus of influenza when penicillin is used.

In conclusion my thanks are due to my colleagues, Mr. Ridley and Mr. Craddock, for their help in carrying out some of the experiments described in this paper, and to our mycologist, Mr. la Touche, for his suggestions as to the identity of the penicillium.

SUMMARY.

1. A certain type of penicillium produces in culture a powerful antibacterial substance. The antibacterial power of the culture reaches its maximum in about 7 days at 20° C. and after 10 days diminishes until it has almost disappeared in 4 weeks.

2. The best medium found for the production of the antibacterial substance has been ordinary nutrient broth.

3. The active agent is readily filterable and the name "penicillin" has been given to filtrates of broth cultures of the mould.

4. Penicillin loses most of its power after 10 to 14 days at room temperature but can be preserved longer by neutralization.

5. The active agent is not destroyed by boiling for a few minutes but in alkaline solution boiling for 1 hour markedly reduces the power. Autoclaving for 20 minutes at 115° C. practically destroys it. It is soluble in alcohol but insoluble in ether or chloroform.

6. The action is very marked on the pyogenic cocci and the diphtheria group of bacilli. Many bacteria are quite insensitive, *e.g.* the coli-typhoid group, the influenza-bacillus group, and the enterococcus.

7. Penicillin is non-toxic to animals in enormous doses and is non-irritant. It doses not interfere with leucocytic function to a greater degree than does ordinary broth.

8. It is suggested that it may be an efficient antiseptic for application to, or injection into, areas infected with penicillin-sensitive microbes.

9. The use of penicillin on culture plates renders obvious many bacterial inhibitions which are not very evident in ordinary cultures.

10. Its value as an aid to the isolation of *B. influenzæ* has been demonstrated.

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